

# Type IV pili function upstream of the Dif chemotaxis pathway in *Myxococcus xanthus* EPS regulation

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## Summary

The developmental bacterium *Myxococcus xanthus* utilizes gliding motility to aggregate during the formation of multicellular fruiting bodies. The social (S) component of *M. xanthus* gliding motility requires at least two extracellular surface structures, type IV pili (Tfp) and the fibril polysaccharide or exopolysaccharide (EPS). Retraction of Tfp is proposed to power S motility and EPS from neighbouring cells is suggested to provide an anchor and trigger for Tfp retraction. The production of EPS in *M. xanthus* is regulated in part by the Dif chemosensory pathway; however, the input signal for the Dif pathway in EPS regulation remains to be uncovered. Using a genetic approach combined with quantitative and qualitative analysis, we demonstrate here that Tfp function upstream of the Dif proteins in regulating EPS production. The requirement of Tfp for the production of EPS was verified using various classes of Tfp mutants. Construction and examination of double and triple mutants indicated that mutations in *dif* are epistatic to those in *pil*. Furthermore, extracellular complementation between various Tfp and *dif* mutants suggests that Tfp, instead of being signals, may constitute the sensor or part of the sensor responsible for mediating signal input into the Dif pathway. We propose that S motility involves a regulatory loop in which EPS triggers Tfp retraction and Tfp provide proximity signals to the Dif pathway to modulate EPS production.

## Introduction

All organisms, large or small, multicellular or unicellular, respond to their environment using signal transduction pathways, the best understood of which is probably the bacterial chemotaxis pathway (Bren and Eisenbach, 2000; Bourret and Stock, 2002; Armitage *et al.*, 2005).

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Signals detected by bacterial chemotaxis systems are frequently soluble chemicals which, upon entry into the periplasmic space, interact with and result in conformational changes of transmembrane chemoreceptors known as methyl-accepting chemotaxis proteins (MCPs). The conformational changes in MCPs result in the modulation of the downstream CheA kinase activity through the mediation of the CheW protein. The phosphate from the CheA autokinase is transferred to the CheY response regulator, which in its phosphorylated form (CheY-P) effects changes in bacterial motility to achieve chemotaxis.

Studies in recent years have considerably expanded both the signals perceived and the processes regulated by bacterial chemotaxis and chemotaxis-like pathways. Such pathways, for example, have been shown to mediate tactic responses to light (phototaxis) (Bhaya, 2004) and oxygen (aerotaxis) (Taylor *et al.*, 1999). The signals for such tactic responses are either intracellular or they can readily reach the cell surface and interact with transmembrane receptors directly or indirectly. In addition to the regulation of taxis, chemotaxis and its homologous systems have now been implicated in the regulation of diverse processes including pili production (Bhaya *et al.*, 2001), flagella biosynthesis (Berleman and Bauer, 2005a), cyst formation (Berleman and Bauer, 2005b), swarm cell differentiation (Burkart *et al.*, 1998), and developmental gene regulation in *Myxococcus xanthus* (Kirby and Zusman, 2003). The signals for the regulation of these less conventional processes are mostly unknown.

The *M. xanthus* Dif chemotaxis-like pathway regulates the production of fibril exopolysaccharides (EPS) (Yang *et al.*, 2000; Bellenger *et al.*, 2002; Black and Yang, 2004), a cell surface component essential for multicellular differentiation and social (S) gliding motility of this developmental bacterium. Under nutrient limitation, *M. xanthus* cells aggregate on surfaces using their gliding motility to form multicellular fruiting bodies. Increasing evidence indicates that *M. xanthus* S motility, as well as bacterial twitching, is powered by the retraction of type IV pili (Tfp) (Kaiser, 2000; Merz *et al.*, 2000; Skerker and Berg, 2001). EPS is proposed to be the anchor and trigger for pilus retraction in *M. xanthus* (Li *et al.*, 2003). The Dif chemotaxis-like proteins have been shown to form part of a signal transduction pathway that regulates EPS production (Yang *et al.*, 2000; Bellenger *et al.*, 2002; Black and Yang, 2004).

DifA, an MCP homologue lacking a prominent periplasmic domain, likely does not bind extracellular ligands directly as do many other MCPs, however. It is unknown what or how signals are detected by the Dif pathway to regulate EPS production in *M. xanthus*.

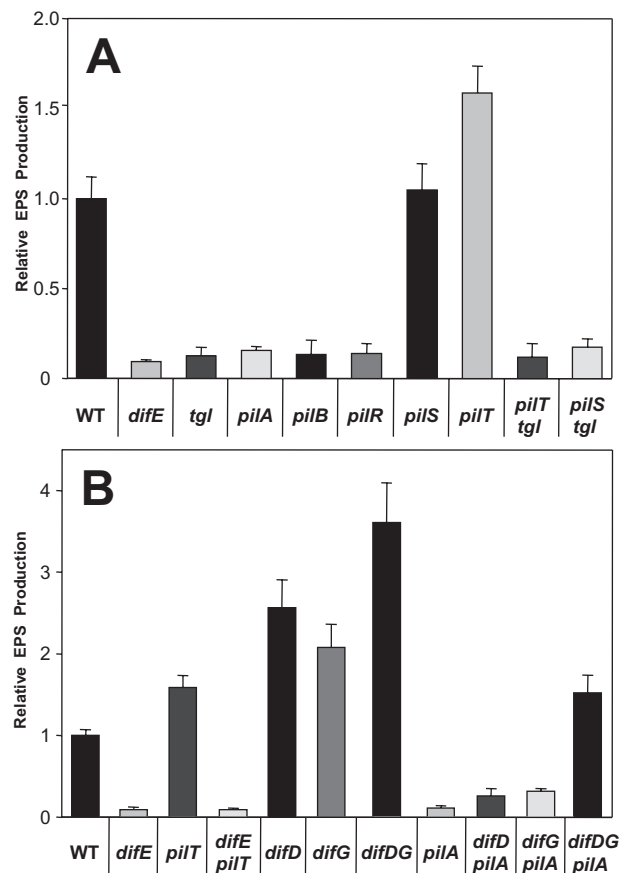
In this study, we show that Tfp, the S motility apparatus, likely function as a sensor or part of a sensor that mediates signal input into the Dif pathway. We first demonstrated that genes required for Tfp biogenesis are required for the production of EPS. Using reciprocal genetic epistasis tests, we provide evidence that Tfp function upstream of the Dif pathway to positively regulate EPS production. In addition, the Dif pathway likely uses DifD, a CheY homologue, as a phosphate sink to divert phosphate from the central Dif pathway in EPS regulation. Extracellular complementation experiments support the hypothesis that Tfp serve in the capacity of sensors instead of extracellular or exogenous signals for the Dif pathway. These results suggest a regulatory loop coupling EPS production to Tfp and vice versa in *M. xanthus*. We propose that Tfp, as extracellular and polarly localized protein filaments, act in essence as sensors to detect cells in their vicinity to regulate EPS production through the Dif chemotaxis-like signalling pathway in *M. xanthus*.

## Results

### *Tfp regulate EPS production*

Dana and Shimkets (1993) observed that many *M. xanthus* S motility mutants failed to bind dyes specific for *M. xanthus* fibril EPS. Some of these mutants are now known to harbour mutations in Tfp biogenesis genes (Wu and Kaiser, 1995), suggesting that Tfp could be essential for EPS production in *M. xanthus*. Examination of various known Tfp mutants confirmed an apparent association between the presence of Tfp and EPS production (Fig. 1A). All mutants (*pilA*, *pilB*, *pilR* and *tgl*) that have no pili (Wu and Kaiser, 1995; Rodriguez-Soto and Kaiser, 1997; Wu *et al.*, 1997) were defective in EPS production. In contrast, *pilS* and *pilT* mutants, both of which assemble pili (Wu and Kaiser, 1997; Wu *et al.*, 1997), still produced EPS. The *pilT* mutant, which is hyperpiliated presumably because it produces non-retractable pili, overproduced EPS when compared with the wild type.

The overproduction of EPS by the *pilT* mutant could be explained by either hyperpiliation or the lack of the PilT protein. A *tgl pilT* double mutant was created to differentiate these two possibilities. Tgl is an outer membrane lipoprotein that is required for Tfp to transverse the outer membrane likely by mediating the assembly of the PilQ secretin multimer (Nudleman and Kaiser, 2004; Nudleman *et al.*, 2005; 2006). The *tgl pilT* double mutant, which lacked Tfp as expected (data not shown), was defective in

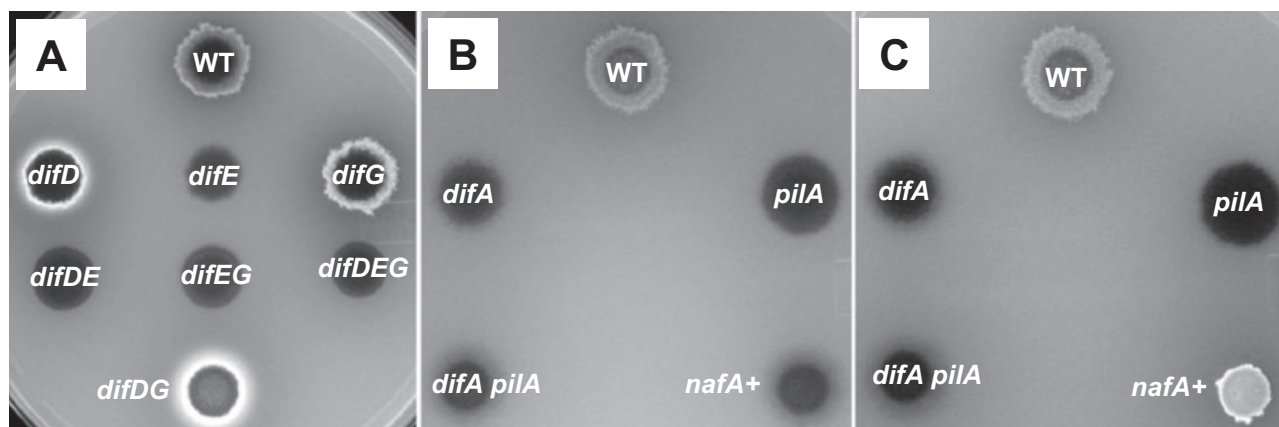


**Fig. 1.** Quantitative analysis of EPS production using Trypan blue binding assay. The amount of dye bound was used as a measurement of EPS production. Values for all strains were normalized to the wild type (see *Experimental procedures*). The data represent three independent experiments, each of which was performed using triplicate samples.  
 A. WT (DK1622), *difE* (YZ603), *tgl* (DK10405), *pilA* (DK10407), *pilB* (DK10416), *pilR* (DK3473), *pilS* (DK10415), *pilT* (DK10409), *pilT tgl* (YZ662) and *pilS tgl* (YZ1602).  
 B. WT (DK1622), *difE* (YZ603), *pilT* (DK10409), *difE pilT* (YZ642), *pilA* (DK10407), *difD pilA* (YZ643), *difG pilA* (YZ645), *difDG pilA* (YZ646).

EPS production (Fig. 1A). This suggests that it is the hyperpiliation, not the loss of PilT *per se*, that is responsible for the observed overproduction of EPS by the *pilT* mutant (Fig. 1A). Likewise, a *tgl pilS* double mutant exhibited the same defects in EPS production as the *tgl* mutant (Fig. 1A). These observations demonstrate that the presence of Tfp structure, retractable or otherwise, is required for EPS production in *M. xanthus*.

### *Tfp function upstream of Dif proteins*

As the *dif* genes play central roles in regulating EPS production, the finding that Tfp are required for EPS production indicates that Tfp functionally interact with the Dif pathway. Genetic epistasis was used to determine whether



**Fig. 2.** Analysis of EPS production by the binding of the fluorescent dye Calcofluor white. Experiments were performed as described in *Experimental procedures*.

A. Indicated strains were spotted on CYE supplemented with Calcofluor white. WT (DK1622), *difD* (YZ613), *difE* (YZ603), *difG* (YZ604), *difDE* (YZ656), *difEG* (YZ657), *difDEG* (YZ658) and *difDG* (YZ641).

B and C. CTT plates supplemented with Calcofluor white without nitrate (B) or with 100  $\mu$ M nitrate (C). WT (DK1622), *difA* (YZ601), *difA pilA* (YZ648) and *nafA+* (YZ729, a *difA pilA* mutant expressing the NafA chimera).

Tfp are downstream or upstream of the known Dif proteins in the regulation of EPS production. If the Dif proteins are downstream of Tfp, *dif* mutations would be epistatic to mutations in *pil* genes. Otherwise, *pil* mutations would be epistatic to mutations in *dif* genes. Various double and triple mutants were constructed and analysed for EPS production to examine the genetic epistatic relationship between *pil* and *dif* mutations (Fig. 1B). A *difE pilT* double mutant showed similar defects in EPS production as the *difE* parental strain, indicating that DifE is likely downstream of Tfp. Previously, *difD* and *difG* mutants were found to overproduce EPS (Black and Yang, 2004) (Figs 1B and 2A). Although a *difD* or *difG* mutation alone only suppressed the EPS defects of a *pilA* mutation minimally if at all (Fig. 1B), a *difD difG pilA* triple mutant produced about 1.5 times the amount of EPS of the wild type (Fig. 1B). These indicate a *difD difG* double mutation can fully suppress a *pilA* mutation with regard to EPS production. These reciprocal genetic epistatic relationships between *dif* and *pil* mutations demonstrated that Tfp function upstream of Dif proteins in the regulation of EPS production.

#### *Neither DifD nor DifG functions downstream of DifE in EPS regulation*

The observation that a *pilA* deletion was fully suppressed only by a *difD difG* double mutation but not by a *difD* or *difG* single mutation (Fig. 1B) could be explained if DifD and DifG function as modulators in the Dif pathway instead of directly downstream of DifE as in one of the two previously proposed models (Black and Yang, 2004). Additional genetic epistasis experiments were performed to examine the functional relationships among DifD, DifE and DifG. As shown in Fig. 2A, neither a *difD* or a *difG*

single mutation nor a *difD difG* double mutation was able to suppress a *difE* mutation. Similarly, previous results had showed that a *difA* deletion could not be suppressed by the deletion of *difD* or *difG* alone or both (Xu *et al.*, 2005). In addition, a *difD difG* double mutant was found to produce more EPS than either the *difD* or the *difG* single parental strain as analysed by the binding of Calcofluor white (Fig. 2A) and by the quantitative EPS assay using Trypan blue (Fig. 1B and Fig. S1), indicating some additive effects by DifD and DifG, both of which were known negative regulators of EPS production (Black and Yang, 2004). All the above results are consistent with a model in which DifD and DifG function as modulators of the activities of the components in the centre of the Dif pathway instead of directly downstream of DifE (Black and Yang, 2004).

#### *Artificial activation of the Dif pathway bypasses the requirement for Tfp*

The interpretation of the epistatic analysis involving *difD* and *difG* mutations (Fig. 1B) became somewhat ambiguous by the above finding that neither DifD nor DifG functions directly downstream of DifE in the regulation of EPS production. The suppression of the defect in EPS production resulting from a *pilA* mutation by mutations in a centre component of the Dif pathway would provide more direct evidence for the proposed functional relationship between Tfp and Dif proteins. A NarX-DifA (NafA) chimera was recently constructed and shown to activate the Dif pathway in a nitrate-dependent manner (Xu *et al.*, 2005). If Tfp are upstream of Dif in regulating EPS production, the artificial activation of the Dif pathway using NafA and nitrate would be predicted to bypass the requirement of



**Fig. 3.** Expression of DifA and DifD in various *pil* mutant backgrounds examined by immunoblotting. Whole cell lysates from  $5 \times 10^7$  were separated by SDS-PAGE and probed with polyclonal antibodies against DifA or DifD.

Tfp. Assays using plates containing Calcofluor white revealed that in the absence of nitrate, YZ729, a *difA pilA* double mutant containing a *nafA* construct, produced no detectable levels of EPS (Fig. 2B); however, in the presence of 100  $\mu$ M nitrate, the same strain clearly produced EPS at levels equivalent to or greater than the wild type (Fig. 2C). These results provided more direct evidence that Tfp function upstream of the Dif pathway in the regulation of EPS production in *M. xanthus*.

A possible and perhaps trivial explanation for the observed epistasis between *dif* and *pil* genes is that Tfp may either downregulate the expression of positive EPS regulators such as DifA or upregulate negative regulators such as DifD. DifA and DifD protein levels in *pil* mutants were examined by immunoblotting and showed no obvious differences from the wild type (Fig. 3). Therefore, the functional interactions between Tfp and the Dif pathway are propagated through signal transduction instead of regulation of *dif* gene expression.

#### *Tfp* or its assembly may function as a sensor for the Dif pathway

How might Tfp function in the signal transduction pathway regulating EPS production in *M. xanthus*? Considering that Tfp are polymeric protein filaments protruding from cell poles, there are at least two possibilities. Tfp may function as extracellular signals of the Dif pathway or Tfp may serve as a sensory apparatus mediating signal input into the pathway. To examine these possibilities, mixing or extracellular complementation experiments were performed using plates containing Trypan blue. Mixing of *difE* and *pilA* mutant cells did not result in EPS production as indicated by the lack of Trypan blue binding (Fig. 4 and Fig. S2). The *difE* mutant, while lacking a functional Dif pathway, is hyperpiliated (Bellenger *et al.*, 2002; Li *et al.*, 2003) whereas the *pilA* mutant lacks Tfp yet possesses a full complement of *dif* genes. If Tfp from neighbouring cells acted solely as external input signals to the Dif pathway, *difE* mutant cells would have provided the signal to the *pilA* mutant cells and stimulated EPS production in the mixed cell population. The lack of EPS production by the mixture of *difE* and *pilA* mutant cells suggests that Tfp do not act merely as extracellular signals to the Dif pathway.

By contrast, if Tfp functions as a sensor or part of a sensor for the Dif pathway, it is conceivable that EPS production could be restored to a Tfp<sup>-</sup> mutant that is extracellularly complemented to assemble Tfp. It is known that Tgl<sup>-</sup> cells, which lack Tfp, can be stimulated to transiently assemble Tfp by close contact with Tgl<sup>+</sup> cells (Wall and Kaiser, 1998; Wall *et al.*, 1998). When *tgl*<sup>-</sup> cells were mixed with either *difE* or *pilA* mutant cells, the mixed population formed bluish-green colonies (Fig. 4 and Fig. S2), indicating the restoration of EPS production. A few lines of evidence support our conclusion that it was the Tgl<sup>-</sup> cells in the mixed population that produced EPS. First, the mixture containing a *difE tgl* double mutant with either a *difE* or a *pilA* mutant failed to produce EPS (Fig. 4), indicating the importance of an intact Dif pathway in Tgl<sup>-</sup> cells in the extracellular complementation. On the other hand, the mixed population of the *difE pilA* double mutant with the *tgl* single mutant produced EPS similarly as the mixture of *pilA* and *tgl* mutants (Fig. 4), indicating that an intact Dif pathway is not necessary in the Tgl<sup>+</sup> donor. Second, it has been shown that pilin, therefore the *pilA* gene, from the Tgl<sup>-</sup> recipient but not from the Tgl<sup>+</sup> donor is required for the stimulation of Tgl<sup>-</sup> cells (Wall *et al.*, 1998). The mixture of *difE* with *pilA tgl* double

	<i>difE</i>	<i>pilA</i>	<i>tgl</i>
<i>difE</i>	na		
<i>pilA</i>	-	na	
<i>tgl</i>	+	+	na
<i>difE tgl</i>	-	-	-
<i>difE pilA</i>	-	-	+
<i>pilA tgl</i>	-	nd	nd
<i>pilT tgl</i>	+	+	nd

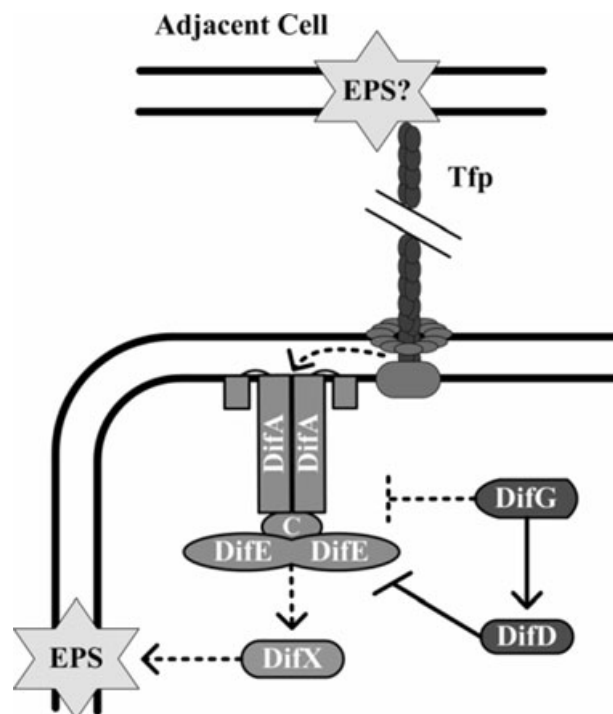
**Fig. 4.** Summary of the mixing experiments or extracellular complementation experiments. Cells of the strains listed on the top were mixed with cells of the strains listed on the left and examined for the binding of Trypan blue, an indicator of EPS production. +, EPS produced; -, EPS not produced; na, not applicable; nd, not determined. *difE* (YZ603), *pilA* (DK10407), *tgl* (DK10405), *difE tgl* (YZ679), *difE pilA* (YZ644), *pilA tgl* (YZ681) and *pilT tgl* (YZ662).

mutant cells did not produce EPS, indicating that the Tgl<sup>-</sup> recipient must have a wild-type *pilA* gene. Lastly, it had been shown previously that adventurous or A motility is essential for the stimulation of Tgl<sup>-</sup> cells to assemble Tfp (Wall and Kaiser, 1998). Our results indicated that the introduction of an A motility mutation into *difE*, *pilA* or *tgl* mutant background abolished EPS production in the extracellular complementation experiments (data not shown). These results are consistent with the interpretation that Tgl<sup>-</sup> cells but not *difE* or *pilA* mutant cells were stimulated to produce EPS when mixed with the appropriate cells. The inference from these results is that the assembly of Tfp by the otherwise Tfp<sup>-</sup> cells of the *tgl* mutant reconstituted a functional sensor for the Dif pathway. This sensor could then detect and transmit signals to the intact Dif pathway downstream leading to EPS production. The observation here also served as a proof of principle for the mixing or extracellular complementation experiments for the examination of EPS production.

The extracellular complementation experiments (Fig. 4) also provided further support for a few conclusions reached earlier. First, the stimulation of *tgl* by either the *pilA* or the *difE pilA* double mutant indicates that external Tfp or lack thereof from the donor cells are not important for EPS production, strengthening the notion that Tfp do not function as external signals for the Dif pathway. Second, we have shown that the *difE tgl* double mutant was stimulated to assemble surface pili when mixed with *pilA* mutants (data not shown). The lack of EPS production by this mutant in the presence of *pilA* mutant cells (Fig. 4) further validate the conclusion that Dif proteins function downstream of Tfp in EPS regulation. Lastly, a *pilT tgl* double mutant retained the ability to produce EPS when stimulated by either *difE* or *pilA* mutant cells (Fig. 4). This observation coincides with the ability of a *pilT* mutant to produce EPS and indicates that non-retractable or paralysed Tfp is sufficient to stimulate the Dif pathway for the regulation of EPS production under our experimental conditions.

## Discussion

In this study, we demonstrated a correlation between the presence of Tfp and the production of EPS in *M. xanthus*. All mutants that fail to assemble pili were found to be defective in EPS production (Fig. 1A) whereas those (*pilS* and *pilT*) that still possess Tfp filaments produced EPS at levels equivalent to or greater than the wild-type strain. Genetic epistasis provided evidence that Tfp function upstream of the Dif chemotaxis-like pathway to positively regulate EPS production (Fig. 1B). Mixing or extracellular complementation experiments indicated that Tfp from adjacent cells could not restore EPS production to Tfp<sup>-</sup>



**Fig. 5.** Model depicting the regulation of EPS production in *M. xanthus* by TFP and the Dif pathway. Demonstrated interactions are indicated by solid lines and proposed interactions by dashed lines. Arrows and bars indicate positive and negative regulation respectively. See text for details of the model.

mutant cells (Fig. 4 and Fig. S2). On the other hand, *tgl* mutants, which can be stimulated extracellularly to produce Tfp transiently (Wall *et al.*, 1998), do produce EPS when mixed with Tgl<sup>+</sup> but EPS<sup>-</sup> cells. In addition, studies of various double mutants showed that DifD (CheY-like) does not function downstream of DifE (CheA-like) in EPS regulation and that DifD and DifG exhibit additive effects as negative regulators of EPS production (Fig. 2A).

### A working model

Based on current and previous studies, a working model is presented in Fig. 5 to describe the regulation of EPS production by the Dif pathway. Tfp are proposed to be at the top of the regulatory cascade and act to mediate signal input into the pathway. The signal is then transmitted to a signalling complex composed of DifA (MCP-like), DifC (CheW-like) and DifE. The DifE putative kinase in this ternary complex is proposed to regulate EPS production in a phosphorylation-dependent manner by transmitting signals downstream to yet unidentified components represented collectively by DifX. DifD directly interacts with DifE as a phosphate sink to negatively regulate EPS production. DifG directly interacts with DifD, presumably as a phosphatase of DifD-phosphate (DifD-P), to promote

the inhibitory effect of DifD on EPS production. DifG is additionally hypothesized to interact with the DifACE complex to negatively regulate the output of the pathway.

This model (Fig. 5) is consistent with and supported by the data currently available. DifA, DifC, DifE and Tfp all positively regulate EPS production whereas DifD and DifG are negative regulators (Yang *et al.*, 2000; Bellenger *et al.*, 2002; Black and Yang, 2004) (Fig. 1). In addition to the epistasis presented in this study, previous studies by the same lab indicated that *difD* and *difG* mutations failed to suppress *difA* mutations in EPS production (Xu *et al.*, 2005). The formation of a DifACE ternary complex was demonstrated using a modified yeast two-hybrid system, as were the interactions of DifD with both DifE and DifG (Yang and Li, 2005). The proposed function of a phosphate sink for DifD, which is supported by genetic epistasis and by the EPS-overproducing phenotype of *difD* mutants, mirrors the function of CheY2 in *Rhizobium meliloti* chemotaxis (Sourjik and Schmitt, 1996; 1998).

DifG must have at least two points of contact with the central pathway. *Bacillus subtilis* CheC, which DifG is homologous to, has been shown to have CheY-P phosphatase activity (Szurmant *et al.*, 2004). Similarly, DifG could simply function as a phosphatase of DifD-P in the Dif pathway in *M. xanthus*; however, this fails to explain the additive effects of *difD* and *difG* mutations on EPS production (Fig. 2A). As *B. subtilis* CheC was observed to interact with both CheA and McpB in yeast two-hybrid experiments (Kirby *et al.*, 2001), we hypothesize that there are additional interactions between DifG and the DifACE ternary complex. The proposed DifACE-DifG interactions are further supported by studies of chemotactic responses of *M. xanthus* to phosphatidylethanolamine (PE). It is known that *difA* and *difC* are essential only for responses to 16:1 PE while *difE* and *difD* are required for responses to both 18:1 and 16:1 PE species (Bonner *et al.*, 2005). Therefore, it appears that multiple upstream pathways responsible for PE tactic responses converge at the point of the DifE kinase. As *difG* is required for chemotactic adaptation to 16:1 but not 18:1 PE (Bonner *et al.*, 2005), the interactions of DifG with the pathway should be restricted to DifA, DifC, and/or other upstream components specific to the 16:1 response.

#### Complex signalling network

*Myxococcus xanthus* Dif pathway is part of a complex signalling network with multiple inputs, distinct outputs, and possible cross-talks with other signalling pathways. There are other known EPS regulators in *M. xanthus* besides the ones depicted in Fig. 5. SglK and Stk, two DnaK homologues, have opposing effects on EPS production (Dana and Shimkets, 1993; Weimer *et al.*, 1998; Yang *et al.*, 1998a). SglK is a positive regulator of EPS

production whereas Stk is a negative one. Some of the 30 plus genes at the *eps* locus clearly encode regulatory proteins rather than biosynthetic ones (Lu *et al.*, 2005). The Dif proteins must somehow interact functionally with these EPS regulators to affect the function of the EPS biosynthetic genes, some of which undoubtedly reside at the *eps* locus (Lu *et al.*, 2005). The Dif pathway is also involved in chemotactic responses to at least two PE species as eluded to earlier (Bonner *et al.*, 2005). In contrast to its role in EPS regulation (Fig. 5), DifD is proposed to function downstream of DifE in the excitation response to these PE molecules (Bonner *et al.*, 2005). FibA, a protein essential for mediating responses to 16:1 PE, does not appear to interfere with the production of fibril EPS (Kearns *et al.*, 2002). On the other hand, the best-known and studied chemotaxis system in *M. xanthus* is encoded by the *frz* genes (Ward and Zusman, 1997). The Dif pathway should interact with the Frz proteins at some level to regulate *M. xanthus* motility behaviour. In addition, while EPS production can be readily analysed under nutrient-rich conditions, starvation is a prerequisite to analyse responses to PE in *M. xanthus* (Kearns *et al.*, 2002). It is not known how nutrient conditions influence the function of the Dif pathway. Regardless of the answers to many of these questions, it is clear that the Dif signalling proteins are involved in multiple processes in a complex regulatory network.

#### *The type IV pilus as a sensor upstream of a chemotaxis-like pathway?*

Why are Tfp required for the regulation of EPS production by the Dif pathway? One possibility is that pilin, the subunit of Tfp, could function as exogenous or endogenous signals for the Dif pathway. The results from extracellular complementation experiments (Fig. 4) are not consistent with pilins from neighbouring cells being exogenous signals. We argue that pilins are unlikely endogenous signals based on the EPS<sup>-</sup> phenotypes of *pilQ* (data not shown) and *tgl* mutants (Fig. 1A). Both Tgl and PilQ are required for the formation of secretin oligomers/channels for the Tfp filaments to traverse the outer membrane (Nudleman and Kaiser, 2004; Nudleman *et al.*, 2005; 2006). *tgl* and *pilQ* mutations were not expected to influence the endogenous pool of pilins. Indeed, *tgl* and *pilQ* mutants contained endogenous pilins indistinguishable in amount and molecular weight from those of the wild type as analysed by immunoblotting of cell lysates prepared from bacteria with their pilus filaments sheared off (data not shown). Pilus retraction is apparently not required for EPS production (Figs 1A and 4), excluding the possibility that retracted pilins could function as endogenous signals. We instead propose that Tfp is the sensor or part of a sensory apparatus that detects and

transmits signals to the Dif pathway (Fig. 5). It was proposed that the distal ends of Tfp may interact with EPS to initiate pilus retraction in *M. xanthus* (Li *et al.*, 2003). We speculate that *M. xanthus* utilizes the distal ends of Tfp to sense the presence of other cells by interacting with EPS on their cell surfaces, and such signals of cell proximity are subsequently relayed to a chemotaxis-like signalling pathway to regulate EPS production in this social bacterium. It appears that not only can Tfp be triggered to retract by EPS (Li *et al.*, 2003), the interactions between these two cell surface structures may also lead to the stimulation of EPS production in *M. xanthus*. Such a regulatory feedback loop makes biological sense considering that *M. xanthus* S motility requires Tfp and EPS as well as cell proximity. There would be no pilus retraction and S motility if there are no adjacent cells to provide EPS within the reach of a pilus (Li *et al.*, 2003); there would be no stimulation of EPS production unless there are nearby cells that can take advantage of these surface molecules.

The current data could also be explained if Tfp assembly, instead of the Tfp filament, functions either as a sensor or a signal for the Dif pathway. This is consistent with the observation that all mutant strains that assemble external Tfp fibres, *pilS*, *pilT* or extracellularly stimulated *tgl* mutants, are proficient in producing EPS (Fig 1 and Fig. S2). On the other hand, we view this as a less favourable explanation partially because of the phenotypes of *pilT* mutants. These mutants, which are hyperpiliated, overproduce EPS as assessed by a quantitative assay (Fig. 1). Our results (Fig. 1A) suggest that it is the hyperpiliation, not the lack of PilT *per se*, that leads to EPS overproduction by these strains. The hyperpiliation of *pilT* mutants is likely due to the lack of retraction, not the assembly of more Tfp (Wu *et al.*, 1997), which argues against Tfp assembly being the sensor or the signal. Nevertheless, it would be premature to rule out this possibility and it is not clear how Tfp, retractable or otherwise, may signal a chemosensory pathway downstream.

#### *Bacterial protein filaments as environmental sensors*

Many bacteria possess cell surface protein filaments that have the potential to function as remote sensors or antennas to monitor their surrounding environments. Flagella have long been suspected to be a sensor of surfaces or viscosity in regulating swarmer cell differentiation (Harshey, 1994; McCarter, 2001). It was suggested more recently that the flagellum could be the sensor of environmental wetness in regulating flagella-based swarming motility (Wang *et al.*, 2005). It was shown recently that a flagellum has the ability to affect the switch in response to changes at its distal end (Fahrner *et al.*, 2003), lending further support to the idea of a remote sensory function for

flagella. Similarly, the type three secretion (T3S) needle, a structure related to flagella, may transmit signals of host contact to trigger the expression and secretion of T3S effectors (Blocker *et al.*, 2003; He *et al.*, 2004; Kenjale *et al.*, 2005). Besides their proposed sensory function in *M. xanthus* by this study, Tfp have been suspected to function in signal transduction in a few other scenarios. It is known that Tfp play critical roles in the colonization of epithelial cells by pathogenic *Neisseria* (Nassif *et al.*, 1999; Koomey, 2001) and enteropathogenic *Escherichia coli* (EPEC) (Giron *et al.*, 1991). It was reported that the transcription of Tfp genes in *Actinobacillus pleuropneumoniae* is regulated by contact with host cells (Boekema *et al.*, 2004). In all cases, Tfp possibly play roles in the sensory transduction of host cell contacts (Merz *et al.*, 1999; Pujol *et al.*, 1999; Tobe and Sasakawa, 2001; Winther-Larsen and Koomey, 2002). Although it has been shown that both the chemotaxis system and the sensory functions of flagella are important for swarmer cell differentiation, the sensory function of flagella likely does not influence or require the chemotaxis signalling network (Fahrner *et al.*, 2003; Wang *et al.*, 2005; Shimizu *et al.*, 2006). This is in contrast to the scenario involving Tfp and Dif in *M. xanthus* in which Tfp clearly function upstream of the Dif chemotaxis-like pathway.

How might a protein filament transmit or propagate signals through the length of the filament to the cell body? It was proposed that the activation signal for T3S could be transmitted mechanically via shifts in the helical architecture of the needle (Blocker *et al.*, 2003). Failure to detect any structural changes or shifts in various secretion mutants also led to the proposal that the signal transduction by T3S needle is through a novel mechanism yet to be discovered (Cordes *et al.*, 2005; Kenjale *et al.*, 2005). Two mechanisms have been proposed for signal transduction by flagella (Fahrner *et al.*, 2003). Mechanical stimulation or loading at the distal end of a flagellar filament could be propagated to the cell body through changes in torque. Alternatively, changes in the speed of motor rotation due to changing load could be sensed by proton flux through the motor complex. It is conceivable that *M. xanthus* Tfp may sense the presence of cells by attachment at the distal end and propagate or transmit the signal mechanically to the cell through structural restraints or shift in the filament (Blocker *et al.*, 2003; Li *et al.*, 2003). It is also possible that the transmission of signals by Tfp is through a novel mechanism yet to be uncovered as suggested for the T3S needle. Whatever the mechanism is, Tfp retraction may not be involved intimately in the process. In this context, it is intriguing to note the roles of Tfp in electron transport in the reduction of insoluble Fe(III) oxides as extracellular electron acceptors in *Geobacter sulphurreducens* (Reguera *et al.*, 2005). Results indicated that *G. sulphurreducens* pili may serve as

nanowires, transferring electrons from the cell to the surface of insoluble Fe(III) oxides at a distance (Reguera *et al.*, 2005). Reminiscent of our results with *M. xanthus pilT* mutants, *G. sulphurreducens* Tfp from a *pilT* mutant could still transfer electrons (Reguera *et al.*, 2005). Interestingly, Tfp filaments from *Shewanella oneidensis* or *Pseudomonas aeruginosa* lacked detectable electron conductivity. The divergence and differences in the properties of Tfp from different organisms may reflect the functional versatility of Tfp that can evolve in organisms from diverse ecological niches. We will continue to investigate how Tfp could sense and transmit signals to a chemotaxis-like pathway in the model organism *M. xanthus*.

## Experimental procedures

### Growth conditions and construction of *M. xanthus* strains

*Myxococcus xanthus* strains were grown and maintained at 32°C on Casitone-yeast extract (CYE) agar plates or in CYE liquid medium (Campos and Zusman, 1975). XL1-Blue (Stratagene), the *E. coli* strain used for plasmid construction, was grown and maintained at 37°C on Luria–Bertani (LB) agar plates or in LB liquid medium (Miller, 1972). Unless noted otherwise, agar plates contained 1.5% agar. When applicable, kanamycin and oxytetracycline were supplemented to media for selection at 100 µg ml<sup>-1</sup> and 15 µg ml<sup>-1</sup> respectively.

DK1622 (Kaiser, 1979) was used as the wild-type and parental strain for all mutants used in this study. DK3473 (*pilR*), DK10405 (*tgl*), DK10407 (*pilA*), DK10409 (*pilT*), DK10415 (*pilS*), DK10416 (*pilB*), YZ601 (*difA*), YZ603 (*difE*), YZ604 (*difG*) and YZ613 (*difD*) have been described elsewhere (Wu *et al.*, 1997; Wall *et al.*, 1998; Black and Yang, 2004; Xu *et al.*, 2005). The *difD difG* double deletion mutant, YZ641, was constructed from YZ613 using pWB119, which contains a *difG* in-frame deletion and a kanamycin-*galK* (KG) cassette used for gene replacement (Ueki *et al.*, 1996; Black and Yang, 2004). The double and triple deletion mutants YZ656 (*difDE*), YZ657 (*difEG*) and YZ658 (*difDEG*) were constructed similarly using *dif* deletion fragments in pBJ113 (Julien *et al.*, 2000). The *pilA* mutation in DK10407 was transferred into the recipient strains YZ613, YZ603, YZ604, YZ641 and YZ601 by Mx4-mediated generalized transduction (O'Connor and Zusman, 1986) to create strains YZ643 (*difD pilA*), YZ644 (*difE pilA*), YZ645 (*difG pilA*), YZ646 (*difDG pilA*) and YZ648 (*difA pilA*) respectively. YZ662 (*pilT tgl*) and YZ1602 (*pilS tgl*) were constructed by Mx4 transduction of the *tgl* mutation from DK10405 into DK10409 (*pilT*) and DK10415 (*pilS*) respectively. YZ642 (*difE pilT*) and YZ679 (*difE tgl*) were generated by homologous integration of a *difE* insertion vector, pYG402 (Yang *et al.*, 1998b), into DK10409 (*pilT*) and DK10405 (*tgl*) respectively. YZ680 (*tgl*) and YZ681 (*pilA tgl*) were generated by homologous integration of pWB520, which contains an internal *tgl* fragment in pZErO-2 (Invitrogen), into DK1622 and DK10407 (*pilA*) respectively. YZ729,

the strain expressing the NarX-DifA (NafA) chimera, was constructed from YZ648 by integration of pXQ719, the *nafA+* construct (Xu *et al.*, 2005), at the Mx8 *attB* site. All newly constructed mutant strains were verified by polymerase chain reaction (PCR), Southern blot (Sambrook and Russell, 2001), and/or phenotypic analyses.

### Examination of EPS production

Two methods were used to examine EPS production. The first method was a qualitative plate assay using the fluorescent dye Calcofluor white (Dana and Shimkets, 1993; Black and Yang, 2004). Briefly, strains to be tested were harvested in exponential phase and resuspended in MOPS (morpholinopropanesulphonic acid) buffer [10 mM MOPS (pH 7.6), 2 mM MgSO<sub>4</sub>] at 5 × 10<sup>9</sup> cells ml<sup>-1</sup>. Five microlitres of this cell suspension was spotted onto CYE or CTT (Kaiser, 1979) plates containing Calcofluor white at a concentration of 50 µg ml<sup>-1</sup>. Plates were incubated for 5 days at 32°C before documentation under long-wavelength (365 nm) UV light.

The second method, a more quantitative one, was a liquid dye binding assay using Trypan blue. All strains tested were harvested at similar culture densities (approximately 3.5 × 10<sup>8</sup> cells ml<sup>-1</sup>). Cells were washed and resuspended in MOPS buffer containing 1 mM CaCl<sub>2</sub> at 2.5 × 10<sup>8</sup> cells ml<sup>-1</sup> and 5 µg ml<sup>-1</sup> of Trypan blue. The assay was then carried out as previously described (Black and Yang, 2004). EPS production of all strains was normalized to that of the wild-type strain which was arbitrarily set as 1.

### Immunoblotting

Cell lysates from 5 × 10<sup>7</sup> cells were separated by SDS-PAGE (10% for DifA gels and 15% for DifD gels) and analysed by immunoblot using standard methods (Sambrook and Russell, 2001). Polyclonal antibodies against DifA (Xu *et al.*, 2005) and DifD (Z. Yang, unpublished data) were used as the primary antibodies.

### Mixing or extracellular complementation experiments

EPS<sup>-</sup> mutants to be tested for extracellular complementation were grown overnight, harvested and resuspended to approximately 5 × 10<sup>9</sup> cells ml<sup>-1</sup> in MOPS buffer. Cell suspensions of two experimental strains were mixed at a 1:1 ratio (Wall *et al.*, 1998). Five microlitres of the cell mixture was spotted onto CYE plates containing Trypan blue (20 µg ml<sup>-1</sup>). Plates were incubated for 5 days at 32°C before scoring for EPS production which is indicated by a bluish-green colour.

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## References

- Armitage, J.P., Holland, I.B., Jenal, U., and Kenny, B. (2005) 'Neural networks' in bacteria: making connections. *J Bacteriol* **187**: 26–36.
- Bellenger, K., Ma, X., Shi, W., and Yang, Z. (2002) A CheW homologue is required for *Myxococcus xanthus* fruiting body development, social gliding motility, and fibril biogenesis. *J Bacteriol* **184**: 5654–5660.
- Berleman, J.E., and Bauer, C.E. (2005a) A che-like signal transduction cascade involved in controlling flagella biosynthesis in *Rhodospirillum centenum*. *Mol Microbiol* **55**: 1390–1402.
- Berleman, J.E., and Bauer, C.E. (2005b) Involvement of a Che-like signal transduction cascade in regulating cyst cell development in *Rhodospirillum centenum*. *Mol Microbiol* **56**: 1457–1466.
- Bhaya, D. (2004) Light matters: phototaxis and signal transduction in unicellular cyanobacteria. *Mol Microbiol* **53**: 745–754.
- Bhaya, D., Takahashi, A., and Grossman, A.R. (2001) Light regulation of type IV pilus-dependent motility by chemosensor-like elements in *Synechocystis* PCC6803. *Proc Natl Acad Sci USA* **98**: 7540–7545.
- Black, W.P., and Yang, Z. (2004) *Myxococcus xanthus* chemotaxis homologs DifD and DifG negatively regulate fibril polysaccharide production. *J Bacteriol* **186**: 1001–1008.
- Blocker, A., Komoriya, K., and Aizawa, S. (2003) Type III secretion systems and bacterial flagella: insights into their function from structural similarities. *Proc Natl Acad Sci USA* **100**: 3027–3030.
- Boekema, B.K., Van Putten, J.P., Stockhofe-Zurwieden, N., and Smith, H.E. (2004) Host cell contact-induced transcription of the type IV fimbria gene cluster of *Actinobacillus pleuropneumoniae*. *Infect Immun* **72**: 691–700.
- Bonner, P.J., Xu, Q., Black, W.P., Li, Z., Yang, Z., and Shimkets, L.J. (2005) The Dif chemosensory pathway is directly involved in phosphatidylethanolamine sensory transduction in *Myxococcus xanthus*. *Mol Microbiol* **57**: 1499–1508.
- Bourret, R.B., and Stock, A.M. (2002) Molecular information processing: lessons from bacterial chemotaxis. *J Biol Chem* **277**: 9625–9628.
- Bren, A., and Eisenbach, M. (2000) How signals are heard during bacterial chemotaxis: protein–protein interactions in sensory signal propagation. *J Bacteriol* **182**: 6865–6873.
- Burkart, M., Toguchi, A., and Harshey, R.M. (1998) The chemotaxis system, but not chemotaxis, is essential for swarming motility in *Escherichia coli*. *Proc Natl Acad Sci USA* **95**: 2568–2573.
- Campos, J.M., and Zusman, D.R. (1975) Regulation of development in *Myxococcus xanthus*: effect of 3':5'-cyclic AMP, ADP, and nutrition. *Proc Natl Acad Sci USA* **72**: 518–522.
- Cordes, F.S., Daniell, S., Kenjale, R., Saurya, S., Picking, W.L., Picking, W.D., et al. (2005) Helical packing of needles from functionally altered *Shigella* type III secretion systems. *J Mol Biol* **354**: 206–211.
- Dana, J.R., and Shimkets, L.J. (1993) Regulation of cohesion-dependent cell interactions in *Myxococcus xanthus*. *J Bacteriol* **175**: 3636–3647.
- Fahrner, K.A., Ryu, W.S., and Berg, H.C. (2003) Biomechanics: bacterial flagellar switching under load. *Nature* **423**: 938.
- Giron, J.A., Ho, A.S., and Schoolnik, G.K. (1991) An inducible bundle-forming pilus of enteropathogenic *Escherichia coli*. *Science* **254**: 710–713.
- Harshey, R.M. (1994) Bees aren't the only ones: swarming in gram-negative bacteria. *Mol Microbiol* **13**: 389–394.
- He, S.Y., Nomura, K., and Whittam, T.S. (2004) Type III protein secretion mechanism in mammalian and plant pathogens. *Biochim Biophys Acta* **1694**: 181–206.
- Julien, B., Kaiser, A.D., and Garza, A. (2000) Spatial control of cell differentiation in *Myxococcus xanthus*. *Proc Natl Acad Sci USA* **97**: 9098–9103.
- Kaiser, D. (1979) Social gliding is correlated with the presence of pili in *Myxococcus xanthus*. *Proc Natl Acad Sci USA* **76**: 5952–5956.
- Kaiser, D. (2000) Bacterial motility: how do pili pull? *Curr Biol* **10**: R777–R780.
- Kearns, D.B., Bonner, P.J., Smith, D.R., and Shimkets, L.J. (2002) An extracellular matrix-associated zinc metalloprotease is required for dilauroyl phosphatidylethanolamine chemotactic excitation in *Myxococcus xanthus*. *J Bacteriol* **184**: 1678–1684.
- Kenjale, R., Wilson, J., Zenk, S.F., Saurya, S., Picking, W.L., Picking, W.D., and Blocker, A. (2005) The needle component of the type III secretion of *Shigella* regulates the activity of the secretion apparatus. *J Biol Chem* **280**: 42929–42937.
- Kirby, J.R., and Zusman, D.R. (2003) Chemosensory regulation of developmental gene expression in *Myxococcus xanthus*. *Proc Natl Acad Sci USA* **100**: 2008–2013.
- Kirby, J.R., Kristich, C.J., Saulmon, M.M., Zimmer, M.A., Garrity, L.F., Zhulin, I.B., and Ordal, G.W. (2001) CheC is related to the family of flagellar switch proteins and acts independently from CheD to control chemotaxis in *Bacillus subtilis*. *Mol Microbiol* **42**: 573–585.
- Koomey, M. (2001) Implications of molecular contacts and signaling initiated by *Neisseria gonorrhoeae*. *Curr Opin Microbiol* **4**: 53–57.
- Li, Y., Sun, H., Ma, X., Lu, A., Lux, R., Zusman, D., and Shi, W. (2003) Extracellular polysaccharides mediate pilus retraction during social motility of *Myxococcus xanthus*. *Proc Natl Acad Sci USA* **100**: 5443–5448.
- Lu, A., Cho, K., Black, W.P., Duan, X.Y., Lux, R., Yang, Z., et al. (2005) Exopolysaccharide biosynthesis genes required for social motility in *Myxococcus xanthus*. *Mol Microbiol* **55**: 206–220.
- McCarter, L.L. (2001) Polar flagellar motility of the *Vibrionaceae*. *Microbiol Mol Biol Rev* **65**: 445–462.
- Merz, A.J., Enns, C.A., and So, M. (1999) Type IV pili of pathogenic *Neisseriae* elicit cortical plaque formation in epithelial cells. *Mol Microbiol* **32**: 1316–1332.
- Merz, A.J., So, M., and Sheetz, M.P. (2000) Pilus retraction powers bacterial twitching motility. *Nature* **407**: 98–102.
- Miller, J.H. (1972) *Experiments in Molecular Genetics*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory.
- Nassif, X., Pujol, C., Morand, P., and Eugene, E. (1999) Interactions of pathogenic *Neisseria* with host cells. Is it possible to assemble the puzzle? *Mol Microbiol* **32**: 1124–1132.

- Nudleman, E., and Kaiser, D. (2004) Pulling together with type IV pili. *J Mol Microbiol Biotechnol* **7**: 52–62.
- Nudleman, E., Wall, D., and Kaiser, D. (2005) Cell-to-cell transfer of bacterial outer membrane lipoproteins. *Science* **309**: 125–127.
- Nudleman, E., Wall, D., and Kaiser, D. (2006) Polar assembly of the type IV pilus secretin in *Myxococcus xanthus*. *Mol Microbiol* **60**: 16–29.
- O'Connor, K.A., and Zusman, D.R. (1986) Genetic analysis of *Myxococcus xanthus* and isolation of gene replacements after transduction under conditions of limited homology. *J Bacteriol* **167**: 744–748.
- Pujol, C., Eugene, E., Marceau, M., and Nassif, X. (1999) The meningococcal PilT protein is required for induction of intimate attachment to epithelial cells following pilus-mediated adhesion. *Proc Natl Acad Sci USA* **96**: 4017–4022.
- Reguera, G., McCarthy, K.D., Mehta, T., Nicoll, J.S., Tuominen, M.T., and Lovley, D.R. (2005) Extracellular electron transfer via microbial nanowires. *Nature* **435**: 1098–1101.
- Rodriguez-Soto, J.P., and Kaiser, D. (1997) The *tgl* gene: social motility and stimulation in *Myxococcus xanthus*. *J Bacteriol* **179**: 4361–4371.
- Sambrook, J., and Russell, D.W. (2001) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.
- Shimizu, T.S., Delalez, N., Pichler, K., and Berg, H.C. (2006) Monitoring bacterial chemotaxis by using bioluminescence resonance energy transfer: absence of feedback from the flagellar motors. *Proc Natl Acad Sci USA* **103**: 2093–2097.
- Skerker, J.M., and Berg, H.C. (2001) Direct observation of extension and retraction of type IV pili. *Proc Natl Acad Sci USA* **98**: 6901–6904.
- Sourjik, V., and Schmitt, R. (1996) Different roles of CheY1 and CheY2 in the chemotaxis of *Rhizobium meliloti*. *Mol Microbiol* **22**: 427–436.
- Sourjik, V., and Schmitt, R. (1998) Phosphotransfer between CheA, CheY1, and CheY2 in the chemotaxis signal transduction chain of *Rhizobium meliloti*. *Biochemistry* **37**: 2327–2335.
- Szurmant, H., Muff, T.J., Ordal, G.W., Bunn, M.W., and Canistraro, V.J. (2004) *Bacillus subtilis* CheC and FliY are members of a novel class of CheY-P-hydrolyzing proteins in the chemotactic signal transduction cascade. *J Biol Chem* **279**: 21787–21792.
- Taylor, B.L., Zhulin, I.B., and Johnson, M.S. (1999) Aerotaxis and other energy-sensing behavior in bacteria. *Annu Rev Microbiol* **53**: 103–128.
- Tobe, T., and Sasakawa, C. (2001) Role of bundle-forming pilus of enteropathogenic *Escherichia coli* in host cell adherence and in microcolony development. *Cell Microbiol* **3**: 579–585.
- Ueki, T., Inouye, S., and Inouye, M. (1996) Positive-negative KG cassettes for construction of multi-gene deletions using a single drug marker. *Gene* **183**: 153–157.
- Wall, D., and Kaiser, D. (1998) Alignment enhances the cell-to-cell transfer of pilus phenotype. *Proc Natl Acad Sci USA* **95**: 3054–3058.
- Wall, D., Wu, S.S., and Kaiser, D. (1998) Contact stimulation of Tgl and type IV pili in *Myxococcus xanthus*. *J Bacteriol* **180**: 759–761.
- Wang, Q., Suzuki, A., Mariconda, S., Porwollik, S., and Harshey, R.M. (2005) Sensing wetness: a new role for the bacterial flagellum. *EMBO J* **24**: 2034–2042.
- Ward, M.J., and Zusman, D.R. (1997) Regulation of directed motility in *Myxococcus xanthus*. *Mol Microbiol* **24**: 885–893.
- Weimer, R.M., Creighton, C., Stassinopoulos, A., Youderian, P., and Hartzell, P.L. (1998) A chaperone in the HSP70 family controls production of extracellular fibrils in *Myxococcus xanthus*. *J Bacteriol* **180**: 5357–5368.
- Winther-Larsen, H.C., and Koomey, M. (2002) Transcriptional, chemosensory and cell-contact-dependent regulation of type IV pilus expression. *Curr Opin Microbiol* **5**: 173–178.
- Wu, S.S., and Kaiser, D. (1995) Genetic and functional evidence that Type IV pili are required for social gliding motility in *Myxococcus xanthus*. *Mol Microbiol* **18**: 547–558.
- Wu, S.S., and Kaiser, D. (1997) Regulation of expression of the *pilA* gene in *Myxococcus xanthus*. *J Bacteriol* **179**: 7748–7758.
- Wu, S.S., Wu, J., and Kaiser, D. (1997) The *Myxococcus xanthus pilT* locus is required for social gliding motility although pili are still produced. *Mol Microbiol* **23**: 109–121.
- Xu, Q., Black, W.P., Ward, S.M., and Yang, Z. (2005) Nitrate-dependent activation of the Dif signaling pathway of *Myxococcus xanthus* mediated by a NarX-DifA interspecies chimera. *J Bacteriol* **187**: 6410–6418.
- Yang, Z., and Li, Z. (2005) Demonstration of interactions among *Myxococcus xanthus* Dif chemotaxis-like proteins by the yeast two-hybrid system. *Arch Microbiol* **183**: 243–252.
- Yang, Z., Geng, Y., and Shi, W. (1998a) A DnaK homolog in *Myxococcus xanthus* is involved in social motility and fruiting body formation. *J Bacteriol* **180**: 218–224.
- Yang, Z., Geng, Y., Xu, D., Kaplan, H.B., and Shi, W. (1998b) A new set of chemotaxis homologues is essential for *Myxococcus xanthus* social motility. *Mol Microbiol* **30**: 1123–1130.
- Yang, Z., Ma, X., Tong, L., Kaplan, H.B., Shimkets, L.J., and Shi, W. (2000) *Myxococcus xanthus dif* genes are required for biogenesis of cell surface fibrils essential for social gliding motility. *J Bacteriol* **182**: 5793–5798.

### Supplementary material

The following supplementary material is available for this article online:

**Fig. S1.** Quantitative analysis of EPS production.

**Fig. S2.** Extracellular complementation experiment to visualize the full summary of data shown in Fig. 3.

This material is available as part of the online article from <http://www.blackwell-synergy.com>