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BrgE is a regulator of *Myxococcus xanthus* development

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Summary

We report here the identification and characterization of a member of the *Myxococcus xanthus* SdeK signal transduction pathway, BrgE. This protein was identified as an SdeK-interacting component using a yeast two-hybrid screen, and we further confirmed this interaction by the glutathione S-transferase (GST) pulldown assay. Additional yeast two-hybrid analyses revealed that BrgE preferentially interacts with the putative amino-terminal sensor domain of SdeK, but not with the carboxy-terminal kinase domain. A *brgE* insertion strain was shown to be blocked in development between aggregation and mound formation, and decreased by 50-fold in viable spore production compared with the parental wild type. These phenotypes are similar to those of *sdeK* mutants. The *brgE* mutation also altered expression of a sample of Tn*5 lac* developmental markers that are also SdeK regulated. Finally, we demonstrated that a *brgE sdeK* double mutant has a more severe sporulation defect than either of the two single mutants, suggesting that BrgE and SdeK act synergistically to regulate wild-type levels of sporulation. In sum, these data suggest that BrgE operates as an auxiliary factor to stimulate the SdeK signal transduction pathway by directly binding to the amino-terminal sensor domain of SdeK.

Introduction

SdeK is a cytosolic two-component histidine kinase required for fruiting body development and sporulation (Garza *et al.*, 1998; Pollack and Singer, 2001). Previously, we demonstrated that SdeK is required for wild-type expression of a variety of early developmental Tn*5 lac* fusions, including Ω4469, Ω4400, Ω4414 and Ω4403, which are activated between 4 and 12 h post initiation by nutrient deprivation (Garza *et al.*, 1998; Pollack and Singer, 2001).

Acting during the same time frame and in conjunction with SdeK is the C-signalling pathway, which is one of the five extracellular signal systems known to be required for *Myxococcus xanthus* development (Hagen *et al.*, 1978; Shimkets *et al.*, 1983). The active C-signal is a 17 kDa protein that is processed from the gene product of *csgA* (Lobedanz and Søgaard-Andersen, 2003). It is associated with the cell surface and stimulates events at the aggregation phase of development (Kim and Kaiser, 1990a; Shimkets and Rafiee, 1990). Like *sdeK* mutants, *csgA* mutants are capable of initiating aggregation, but fail to progress to the translucent mound stage and are defective for both mature fruiting body formation and sporulation (Kim and Kaiser, 1990b; 1991). In addition, C-signalling mutants are defective for expression of many of the same Tn*5 lac* fusions regulated by SdeK, including Ω4414, Ω4400 and Ω4403 (Kim and Kaiser, 1990b; Pollack and Singer, 2001), but not the Ω4469 fusion (Pollack and Singer, 2001).

Given their positions as regulators of early, aggregation-associated events in development, SdeK, CsgA and additional components acting in their respective pathways likely play pivotal roles in the ‘decision-making events’ before the earliest observed morphological changes. Thus, our understanding of early developmental events would benefit from the identification of more of these components. Several components in the C-signalling pathway have been previously identified and characterized, including FruA (Ogawa *et al.*, 1996; Ellehauge *et al.*, 1998), ActABCD (Gorski and Kaiser, 1998; Gorski *et al.*, 2000; Gronewold and Kaiser, 2001; 2002) and the Frz system (Søgaard-Andersen *et al.*, 1996). Here, we report the use of a yeast two-hybrid screen to identify a component of the SdeK pathway, BrgE. The data presented here demonstrate that BrgE interacts with SdeK to regulate events early in *M. xanthus* development.

Results

Identification of BrgE by yeast two-hybrid screen

The *Saccharomyces cerevisiae* Gal4p transcriptional activator is composed of a DNA-binding domain (BD) and an RNA polymerase-recruiting activation domain (AD). A yeast two-hybrid approach devised by James *et al.* (1996) splits the two domains into separate proteins, Gal4 BD

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and Gal4 AD, which are individually incapable of activating Gal4-dependent promoters. However, if Gal4 BD and Gal4 AD are each separately fused to other proteins that are binding partners, then the two Gal domains would be brought into close enough proximity to restore Gal4p function. In this assay, Gal4p function is determined by the activation of three reporter genes: *ADE2*, *HIS3* and *lacZ*.

Using an SdeK–Gal4 BD fusion as bait, the yeast two-hybrid method was applied to identify components interacting with SdeK (see *Experimental procedures*). Of 100 000 interactions screened, only six candidate SdeK binding partners fully restored Gal4p function (for a complete list, see *Experimental procedures*). One of the strongest candidates was the fusion protein encoded on the yeast two-hybrid plasmid pDV404, which was determined by sequence analysis to be homologous to Baf, a component involved in the regulation of *Bordetella pertussis* virulence genes via the BvgAS two-component system (DeShazer *et al.*, 1995; Wood and Friedman, 2000). On the basis of this homology and its regulatory function in development (see below), we named this protein BrgE for Baf-like regulatory Effector.

Biochemical interactions between BrgE and SdeK

SdeK is composed of two domains, an amino (N)-terminal domain (NTD) and a carboxy (C)-terminal domain (CTD). The NTD contains a PAS domain, flanked by two PAC domains. PAS domains are reported to be associated with environmental sensory functions, such as detecting fluxes in light, redox potential, oxygen, small ligands and the overall cellular energy state (for a review, see Taylor and Zhulin, 1999). The CTD of SdeK contains the phosphotransferase H-box domain, which includes the conserved histidine at position 286, and an ATPase domain involved in cross-phosphorylation (Pollack and Singer, 2001). The BrgE construct was tested separately against yeast two-hybrid constructs containing either the NTD or CTD of SdeK to determine with which it interacts. The results show that there is no detectable productive interaction between BrgE and the CTD of SdeK, while the interaction between BrgE and $SdeK_{NTD}$ is capable of activating the *HIS3* and *lacZ* reporter genes, but not the *ADE2* reporter gene (Table 1). The *ADE2* reporter is considered the most stringent phenotypic test among the three examined (James *et al.*, 1996), and therefore, its inactivity in strain TII 159 suggests a reduced strength for the BrgE–$SdeK_{NTD}$ interaction compared with the interaction between BrgE and full-length SdeK, which activates all three reporter genes (Table 1).

When the reciprocal BrgE–SdeK interaction was tested by switching the fusions, none of the three reporter genes were activated. Such non-reciprocality, reported in the literature for interactions confirmed by other methods

Table 1. Yeast two-hybrid interactions *Between* BrgE and SdeK constructs.

		Reporter phenotype		
Strain	Interaction examined	Lac[a]	His[b]	Ade[b]
TII-160	BrgE and SdeK	+++	+	+
TII-159	BrgE and $SdeK_{NTD}$	++	+	–
TII-161	BrgE and $SdeK_{CTD}$	–	–	–
TII-162	BrgE and vector	–	–	–

a. Determined by whether cells developed blue coloration on non-selective SC agar plates containing X-Gal. Intense blue colour development is represented by +++, light blue colour development by ++, and little or no colour development by –.
b. Complementation of auxotrophies was determined by the growth of cells on SC media lacking histidine or adenine.

(Estojak *et al.*, 1995; Fury *et al.*, 1997), may indicate that the conformation of either BrgE or SdeK, or of both, is/are altered when fused to a different Gal4 domain and that this conformational change disallows a favourable interaction between the partners.

To corroborate the yeast two-hybrid findings by an independent biochemical method, we constructed a glutathione S-transferase (GST)–BrgE fusion and assessed its ability to bind ^{32}S-labelled SdeK by the GST pulldown assay (Kaelin *et al.*, 1991). Compared with GST alone, GST–BrgE pulled down 10-fold more labelled SdeK, which is approximately 55 kDa in size (Fig. 1). These results were consistently reproducible across six individual replicate experiments, and did not appear to be affected significantly by the relative amounts of GST–BrgE and GST bait proteins used. That is, in all cases, at least 10-fold more labelled SdeK was pulled down by GST–BrgE than by GST alone, even in experiments when the amount of GST control protein used was twofold higher than GST–BrgE.

BrgE sequence and locus

Using the recently available *M. xanthus* genome sequence, a single locus was identified corresponding to the sequence of the pDV404 insert. The 771-base pair (bp) *brgE* open reading frame (ORF) (TIGR designation: MXAN4151) is predicted to encode a 256-amino-acid protein with 36% identity and 50% similarity to the *B. pertussis* Baf protein (Fig. 2). BrgE-like proteins are found in many other bacteria, including the uncharacterized YacB protein in *Bacillus subtilis* (Fig. 2). Interestingly, the genome of *Escherichia coli* does not appear to encode for a BrgE-like protein. Available domain prediction programs such as SMART analysis (Schultz *et al.*, 1998; Letunic *et al.*, 2002) and PSI-BLAST analysis (Altschul *et al.*, 1997) were used to aid in predicting the function of BrgE; however, no conserved domains could be identified by this approach. In addition, a BLAST search of the entire

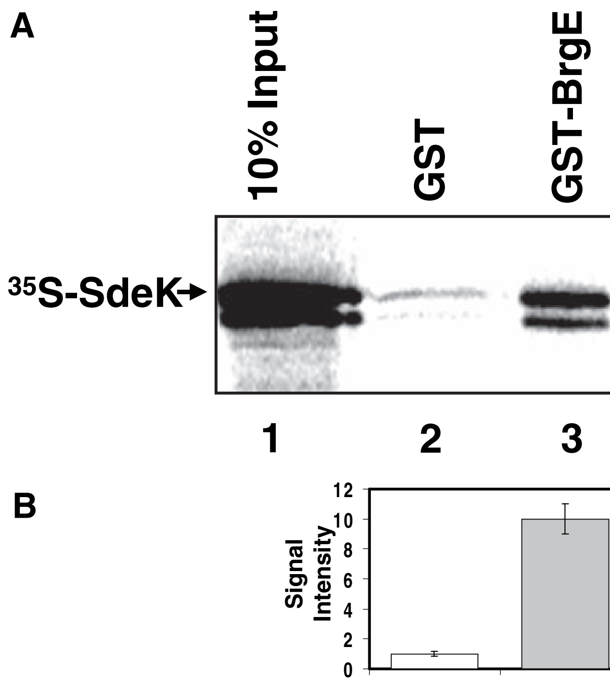

Fig. 1. GST pulldown assay using GST–BrgE or GST as bait for binding ^{35}S-labelled SdeK protein (55 kDa).
A. *In vitro* translated ^{35}S-labelled SdeK protein was pulled down by either 5 μg of GST–BrgE or 10 μg of GST protein, and separated by 10% SDS-PAGE (lane assignments: 1, 10% of ^{35}S-labelled SdeK input; 2, GST; 3, GST–BrgE). The smaller of the two bands is most likely the product of an internal start codon.
B. Signal strength, with arbitrary units, of ^{35}S-labelled SdeK pulled down by bait proteins from six independent replicate experiments.

M. xanthus genome indicated that BrgE is the only Baf or Baf-like homologue in *M. xanthus* that passed the Expect (E) value threshold of 10^{-4}.

Examination of the *brgE* locus revealed that *brgE* is located near the *frz* region (Fig. 3). Upstream of *brgE* is a 1005 bp ORF (MXAN4152) bearing homology (31% identity and 48% similarity at the amino acid level) to the *E. coli birA* gene. In *E. coli*, BirA is a bifunctional protein that both catalyses the ligation of a biotin moiety onto a specific lysine residue of the biotin carboxyl carrier protein, and represses the biotin biosynthetic operon by binding to an operator site (for a review, see Chapman-Smith and Cronan, 1999). Only 61 bases separate the *M. xanthus birA* gene from *brgE*, which suggests transcriptional coupling of the two genes. We attempted to make an in-frame deletion of *birA* by allelic exchange using the GalK selection method of Ueki *et al*. (1996). Although we were able to target the plasmid containing the deletion construct to the *birA* locus, following GalK selection, only the wild-type allele was recovered after 28 independent attempts. These data strongly suggest that a deletion of *birA* is a lethal mutation in *M. xanthus*, as is the case for *E. coli* (Barker and Campbell, 1980; Campbell *et al*., 1980).

Downstream of *brgE* is an 1134 bp ORF (MXAN4150) that does not match any known genes in the sequence databanks. This hypothetical gene was previously predicted by Ward *et al*. (2000) to lie downstream of *frzS*, and to be transcribed in the convergent direction. Reverse transcription polymerase chain reaction (RT-PCR) analyses reveal that the transcription of this gene is not affected

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B.pertussis Baf    MIILLIDSGNSRLKVGWEDPDAPQAAREPAPVAFDNLDLDALGRWLATLPRRPQRALGVNV 60
M.xanthus BrgE     MLLAIDVGNTNTVLGVFEGRRLLDHWRVETSTRR--TSDEYGILVRQLFTHRGIDPMKVT 58
B.subtilis YacB    MLLVIDVGNTNTVLGVYHDGKLEYHWRIETSRHK--TEDEFGMILRSLFDHSGLMFEQID 58

B.pertussis Baf    AGLARGEAIAATLRAGGCDIRWLRAQPLAMG------LRNGYRNPDQLGADRWACMVGVL 114
M.xanthus BrgE     AVVVSSVVPPLQSNLEKMSERYFRVRPMFIGPGVKTGMPILYDNPREVGADRIVNAVSAY 118
B.subtilis YacB    GIIISSVVPPIMFALERMCTKYFHIEPQIVGPGMKTGLNIKYDNPKEVGADRIVNAVAAI 118

B.pertussis Baf    ARQPSVHPPLLVASFGTATTLDTIGPDNVFPGGLILPGPAMMRGALAYGTAHLPLADGLV 174
M.xanthus BrgE     ERH---HAGVLVVDFGTATTFDAVSPKGEYLGGCICPGINISMEALFQNASKLPRVEFAR 175
B.subtilis YacB    HLY---GNPLIVVDFGTATTYCYIDENKQYMGGAIAPGITISTEALYSRAAKLPRIEITR 175

B.pertussis Baf    ADYPI--DTHQAIASGIAAAQAGAIVRQWLAGRQRYGQAPEIYVAGGGWPEVRQEAERLL 232
M.xanthus BrgE     PPHVIGRNTVHSMQAGLVYGYVGMVDGICARMQAELGFPVKVVATGGLASLVASESKAIH 235
B.subtilis YacB    PDNIIGKNTVSAMQSGILFGYVGQVEGIVKRMKWQA----------------KQDPRSLR 219

B.pertussis Baf    AVTGAAFGATPQPTYLDSPVLDGLAALAAQGAPTA 267
M.xanthus BrgE     QVD-------------EFLTLEGLRIIYGRNHAS- 256
B.subtilis YacB    QEA-------------WRRSLRTNQIV-------- 233
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Fig. 2. Amino acid sequence alignment of BrgE with Baf from *Bordetella pertussis* and YacB from *Bacillus subtilis* using CLUSTALW (http://www.ebi.ac.uk/clustalw/). Conserved residues are shaded in black to denote identity, dark grey to denote strong similarity or light grey to denote 'semi-conservation' as defined by the CLUSTALW program.

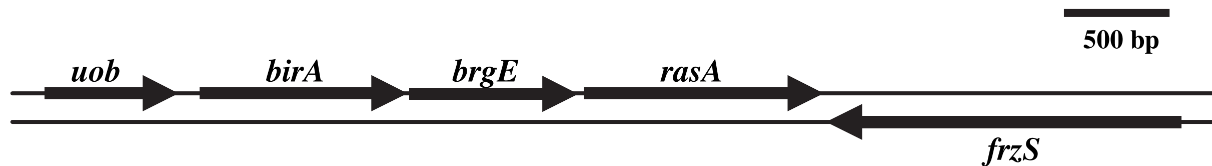

Fig. 3. Map of *brgE* chromosomal region. ORFs are represented as arrows pointing in the presumed direction of transcription. *uob*, for upstream of *birA*, is a temporary designation for an ORF having no homology above an Expect (E) value of 0.030 on the NCBI BLAST database (http://www.ncbi.nlm.nih.gov/BLAST/).

by the *brgE* mutation (data not shown). A phenotypic characterization of a mutant for this gene, which we named *rasA* for required for aggregation and social motility, reveals that it is completely incapable of initiating development and produces no detectable levels of spores (V.D. Pham, C.W. Shebelut, B. Mukherjee, and M. Singer, unpublished data); this is in contrast to the *brgE* mutant, which forms loose mounds and sporulates at reduced frequencies relative to wild type (see below). Moreover, unlike *brgE* cells, the *rasA* mutant is strongly defective for extracellular fibrils needed in social (S)-motility. Based on these phenotypic properties, it is unlikely that *brgE* and *rasA* are functionally related.

An insertion in brgE *causes defects for fruiting and sporulation*

When assessed for developmental competency compared with the parent wild type, the *brgE* insertion strain forms loose aggregates and mounds that never condense or darken into mature-looking fruiting bodies (Fig. 4). Rather, they persist as loose and elongated assemblages of cells more than a week into development, similar to *sdeK* mutants. The *brgE* insertion mutant is also defective for spore viability, with only 2.26% viable spores produced compared with wild type, which is a weaker effect than for mutations in *sdeK* or *csgA* (Table 2).

The brgE *mutation alters expression of some developmental genes*

SdeK-dependent fusions Ω4469, Ω4403, Ω4414 and Ω4400 were analysed for their dependence on BrgE. The rationale is that if BrgE functions in the SdeK-dependent pathway, mutations in BrgE should affect SdeK-dependent fusions in a manner qualitatively similar to that of *sdeK* mutants. These results are shown in Fig. 5. First, the *brgE* insertion causes a twofold increase in the expression of Ω4469 (Fig. 5A), which is identical to the effect observed for an *sdeK* mutant (Pollack and Singer, 2001). Second, we observed a three- to fourfold decrease in the expression patterns of the Ω4414 and Ω4403 fusions (Fig. 5B and C), both of which are also dependent on SdeK and CsgA to a similar extent (Kroos and Kaiser, 1987; Pollack and Singer, 2001). In contrast, BrgE does not appear to participate with SdeK in regulating Ω4400 (Fig. 5D). This result suggests that BrgE function is not necessary for regulating all SdeK-dependent events.

SdeK and BrgE act synergistically to control sporulation

To further characterize the role of BrgE in early development, we constructed *brgE sdeK* and *brgE csgA* double mutants and assayed these strains for effects on fruiting body formation and sporulation. The data demonstrate that both the *brgE sdeK* and *brgE csgA* double mutants display a similar fruiting body phenotype to the *sdeK* and *csgA* single mutants (Fig. 4). With respect to sporulation, the *brgE csgA* double mutant, like the *csgA* single mutant, also produces no detectable levels of viable spores (Table 2). In contrast, the *brgE sdeK* double mutant is more severely defective than either *sdeK* or *brgE* single mutants by three and four orders of magnitude, respectively (Table 2), strongly suggesting a model wherein BrgE and SdeK work in concert to drive sporulation.

brgE *and* sdeK *mutations cause nearly identical predation phenotypes*

We recently developed an assay to measure the effect of various mutations on the predation ability of *M. xanthus* (Pham *et al*., 2005). The data from this study led us to conclude that early developmental components, including SdeK, are required for efficient predation. In particular, we found that the earlier in development a protein functions, the more important its role appears to be for predation. Thus, a mutation in AsgA, which is one of the earliest

Table 2. Spore viability assay.[a]

Genotype	Viable spores[b]	% Wild type (mean ± range)
Wild type	1.4×10^7	100.0 ± 23.5
brgE	3.4×10^5	2.26 ± 0.32
sdeK	3.7×10^4	0.26 ± 0.05
brgE sdeK	<10[c]	$<10^{-4}$
csgA	<10	$<10^{-4}$
brgE csgA	<10	$<10^{-4}$

a. Spores were harvested at 72 h after development was initiated by spotting 5×10^8 cells onto TPM starvation agar.
b. Values shown represent the means of three independent experiments.
c. Spore counts below 10 per experiment were undetectable by our methods.

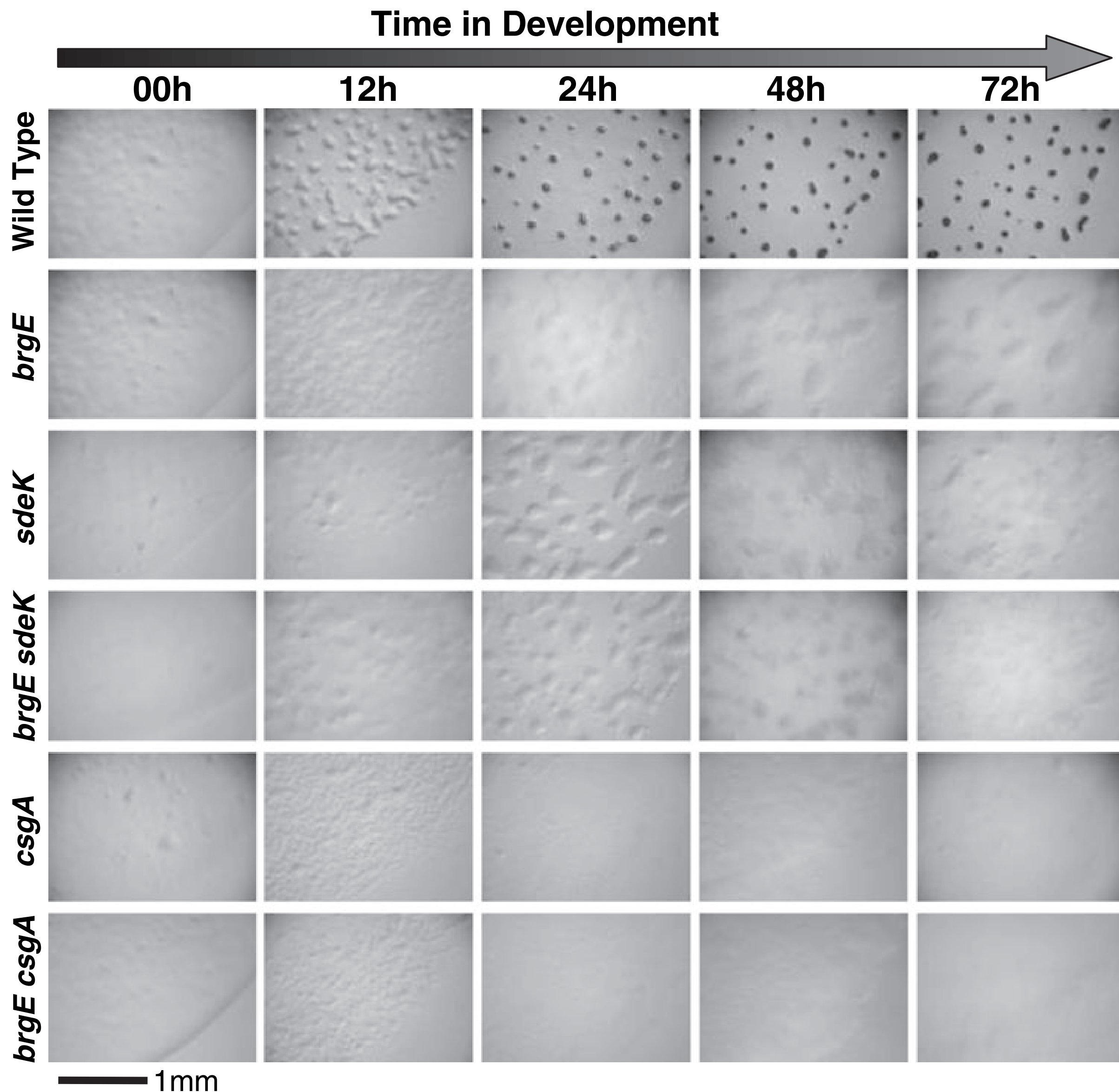

Fig. 4. Developmental progression of wild-type cells and various mutants on TPM starvation agar during a 72 h time-course. Strains used were DK1622 (parent wild type), VP664 (*brgE*), DK7862 (*sdeK*), VP1014 (*brgE sdeK*), DK5208 (*csgA*) and VP825 (*brgE csgA*).

developmental regulators, decreases the predation ability of cells to about 27% of wild type, while mutations in developmental components important for aggregation, e.g. CsgA, causes a more modest decrease to about 64–67% of wild type. When *brgE* and *sdeK* mutants were assayed for predation, the data show that they have almost identical predation defects, at about 65% of wild type, with standard deviations of 2–5%.

Discussion

Previously, we demonstrated that the SdeK pathway is a critical regulatory component of the *M. xanthus* developmental process, and that, along with the C-signalling pathway, it co-ordinates the activation of developmental gene expression during aggregation (Pollack and Singer, 2001). The SdeK regulon includes the sporulation-essential *devTRS* locus (formerly, *4414*; Thöny-Meyer and Kaiser, 1993; Boysen *et al*., 2002), the nutrient sensing *nsd* gene (formerly, *4469*; Brenner *et al*., 2004), as well as other developmentally regulated genes without deduced functions (e.g. *4400* and *4403*) (Pollack and Singer, 2001). Little is known about the components that directly interact with SdeK to form this signal transduction pathway. One protein that is hypothesized to exist is the partner response regulator, which, upon being phosphorylated

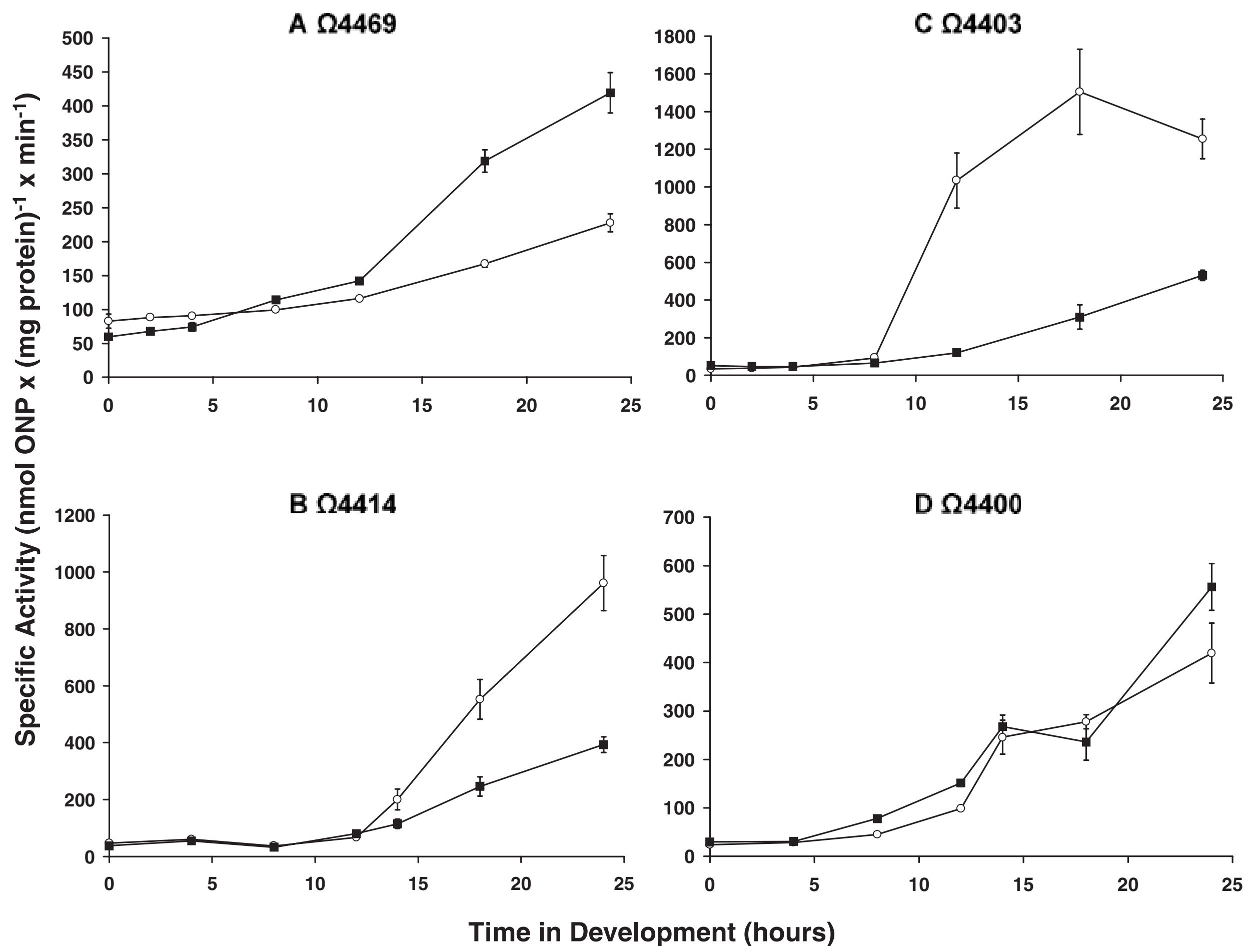

Fig. 5. Effect of the *brgE* insertion mutation on regulation of four developmental Tn*5 lac* fusions: (A) Ω4469, (B) Ω4414, (C) Ω4403 and (D) Ω4400. Wild-type (–■–) and *brgE* (–○–) cells were induced to undergo development on TPM starvation agar. Threefold replicate samples were harvested at various time points between 0 and 24 h into development, and β-galactosidase activity was monitored by the production of *o*-nitro-phenol (ONP).

through direct contact, would carry out the effector functions of a classical histidine protein kinase like SdeK. Here, we described a yeast two-hybrid screen using SdeK as bait in an attempt to identify this predicted component. Although the screen was not saturating, it did succeed in identifying an interacting component we are designating BrgE.

BrgE is only the second member of the Baf-like family of proteins to be characterized. First studied in *B. pertussis*, the aetiologic agent for whooping cough, Baf appears to interact with a two-component system called BvgAS to regulate transcription of *ptxA*, which encodes for the S1 subunit of pertussis toxin (DeShazer *et al*., 1995; Wood and Friedman, 2000). In addition, Baf is essential in *B. pertussis*, implying it regulates additional genes not yet examined (DeShazer *et al*., 1995). In contrast to Baf, BrgE is not essential for growth in *M. xanthus*, although the data above show that it is important for development. Like Baf, however, BrgE also interacts with a two-component signal transduction system, represented by SdeK.

Relationship between BrgE and products of adjacent genes

The *brgE* gene is flanked by two other genes that are predicted to be transcribed in the same direction. One, *rasA*, is located downstream of *brgE* and appears to have no homology to characterized genes in sequence databases. Unlike the *brgE* mutant, *rasA* cells are completely blocked in S-motility, as well as development. It is, thus, not clear whether any functional relationship exists between BrgE and RasA; however, studies are currently being conducted to examine this further. Located upstream of *brgE* is a putative homologue of the *E. coli birA* gene. The *E. coli* BirA acts as a transcriptional repressor by binding to an operator site in the promoter region of the divergently transcribed *bioA* and *bioBCDF* operons (Barker and Campbell, 1981; Barker *et al*., 1981; Lin *et al*., 1991). In addition, it also functions as a biotin protein ligase by catalysing both the synthesis of biotinyl-5′-adenylate and the transfer of the biotin prosthetic group

to biotin carboxyl carrier protein (Chapman-Smith and Cronan, 1999). The *E. coli* BirA protein possesses three discrete domains, with an amino-terminal DNA BD – absent in the *M. xanthus* BirA – required for its repressor function, and central and carboxy-terminal domains important in its catalytic functions (Xu and Beckett, 1996; Kwon *et al.*, 2000; Chapman-Smith *et al.*, 2001). Our study strongly suggests that a deletion of the *M. xanthus* BirA homologue is lethal, which is in line with the demonstrated essentiality of the biotin protein ligase function of BirA in *E. coli* (Barker and Campbell 1980; Campbell *et al.*, 1980). However, as we did not attempt the *birA* deletion in a strain also bearing a wild-type copy of *birA* placed at a heterologous site, it cannot be ruled out that our inability to generate a deletion may result from a problem originating in our plasmid construct.

It is uncertain what, if any, relationship exists between the biotinylation function of BirA and the regulatory role of BrgE in development. A functional relationship is suggested by the BirA homologue in *Neisseria meningitidis*, which is actually a fusion protein composed of conserved BirA domains, plus an additional domain that is homologous with BrgE (Parkhill *et al.*, 2000; Tettelin *et al.*, 2000). It is possible that BrgE in *M. xanthus* cooperates with BirA to mediate the initiation of development when biotin levels, as reported by BirA biotinylation activity, become limiting due to nutrient depletion. However, the validation of this hypothesis awaits further examination of how biotin levels and BirA catalytic activity are related to the functions of BrgE and SdeK.

Relationship between BrgE and CsgA

In this study, our analyses were focused on testing the hypothesized relationship between BrgE and SdeK. Another regulatory pathway, the C-signal-dependent pathway, in which CsgA is the most well-characterized component, is known to be active at the same time as the SdeK pathway. Because the *csgA* mutation is epistatic to the *brgE* mutation, this suggested that either BrgE operates upstream of CsgA, or BrgE is in a pathway that functions in parallel with that of CsgA, with the *csgA* mutation causing more dominant fruiting body morphology and sporulation phenotypes. A model has been previously proposed whereby the SdeK and C-signal pathways act in parallel during the early stages of development, then converge at aggregation to regulate developmental genes under their common, dual control (Julien *et al.*, 2000; Pollack and Singer, 2001). Pending further investigations, the Ω4469 regulation data position BrgE only within the SdeK developmental pathway (Fig. 6).

Relationship between BrgE and SdeK

Several lines of evidence support an affiliation for BrgE

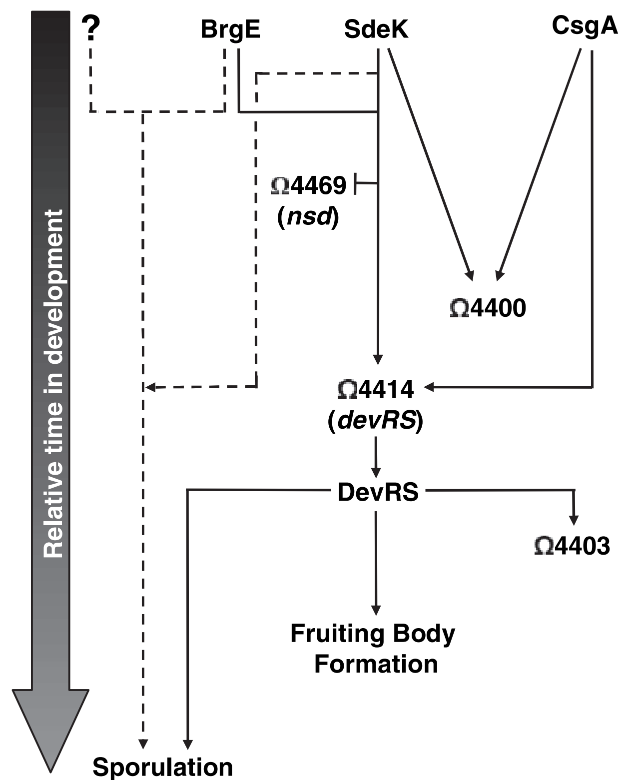

Fig. 6. Model illustrating the relationships among BrgE, SdeK, C-signalling and known components of the SdeK regulon during development. Stimulation of activity is represented by a pointed arrow, while inhibition is represented by a perpendicular bar. BrgE interacts with SdeK to drive expression of some developmental Tn*5 lac* reporter fusions and fruiting body formation. Separately, BrgE is hypothesized to interact with an unidentified third component (represented by a '?') that regulates, in concert with SdeK, a pathway necessary for sporulation (broken arrows).

with the SdeK pathway. First, *brgE* and *sdeK* mutants cause similar effects on a sample of Tn*5 lac* transcriptional fusions, including increased expression of the Ω4469 fusion at the *nsd* locus, which heretofore had been known to be regulated only by SdeK (Pollack and Singer, 2001). Second, the predation assay data show that BrgE and SdeK are equally important for predation, which suggests a close functional partnership between the two proteins for matters relating to nutrient scavenging. Third, as determined by both yeast two-hybrid assay and GST pulldown assay, BrgE directly interacts with SdeK.

Finally, the effects on fruiting body formation by the *brgE* mutant are similar to those of the *sdeK* mutant, although the *brgE* mutant phenotype is quantitatively less severe (by about 10-fold) with respect to sporulation. This implies that SdeK is able to carry out some regulatory functions in the absence of BrgE, which is supported by the fact that BrgE plays no role in the regulation of the SdeK-dependent fusion Ω4400 (Fig. 5D). However, our data for the *brgE sdeK* double mutant suggests that the interaction of these two proteins is much more complex: although a

Table 3. Sequences of oligonucleotide primers.

Primer	Sequence (5′ to 3′)[a]	Purpose
BrgE-F	GCCTGCAGAATACCGTGCTCGGAGTGTTC	*brgE* insertional disruption
BrgE-R	GCCGAATTCCATGCCCACGTAGCCGTAGAC	
BirA1	CCGAAGCTTAGGCCTATCTCTACACGATGG	*birA* in-frame deletion (upstream amplicon)
BirA2	GGACTGCAGATGGCGGAGAAGTAGAGATTG	
BirA3	GGACTGCAGGTTGGACCTCTACCTGGACAC	*birA* in-frame deletion (downstream amplicon)
BirA4	ATGGAATTCGATTCCTTCCGTAGATGATGC	
SdeK-F19	GGGAATTCATCGGGGGCCACGCCGACATGTCATCT	Yeast two-hybrid fusion constructs[b]
SdeK-F826	GGGAATTCTTCCAGGAGCGCTTCATCGGCGTGCTG	
SdeK-R852	GGAGATCTCAGCACGCCGATGAAGCGCTCCTGGAAG	
SdeK-R1506	GGAGATCTGGTGACGCGAGGCAGCGTCACCGAGAAG	

a. Restriction enzyme sites engineered into each primer are underlined. Sites are: AGATCT for *Bgl*II, GAATTC for EcoRI, AAGCTT for *Hin*dIII and CTGCAG for PstI.
b. SdeK-F19 and -R1506 were used to amplify nearly the full length of *sdeK*, SdeK-F19 and -R852 were used to amplify the portion encoding for the SdeK amino-terminal domain, and SdeK-F826 and -R1506 were used to amplify the portion encoding for the SdeK carboxy-terminal domain.

small number of spores is produced by both the *brgE* and the *sdeK* single mutants, spore production for the *brgE sdeK* double mutant is decreased by three to four orders of magnitude and is virtually undetectable (Table 2). Additive phenotypes such as these are typically interpreted as evidence for the convergence of two parallel pathways. The simplest interpretation of the data is that BrgE also functions in a second pathway that is involved in sporulation, which acts synergistically with SdeK; this hypothetical model is diagrammed in Fig. 6. In this model, the absence of input from either BrgE or SdeK would allow the sporulation pathway to function at least partially, but sporulation would be completely shut off if neither component is present.

An extension of this model is the prediction that the BrgE–SdeK co-regulation of sporulation is not mediated through DevRS (4414) alone, which is supported by the observation that *devRS* mutants still produce a small number of spores (Ellehauge *et al.*, 1998). Thus, while a summation of the data supports a model whereby many of the regulatory functions of BrgE are mediated through its interaction with SdeK, particularly with regards to fruiting body development (Fig. 6), their partnership vis-à-vis sporulation requires further examination. For this purpose, we will be using microarrays to identify the remaining components in the SdeK- and BrgE-associated pathways. By revealing the rosters of their respective regulons, these analyses are expected to provide a global perspective for the regulatory functions of both proteins.

Experimental procedures

Strains, culturing conditions and motility assessments

The strains used in this study are listed on Table 4. *E. coli* strains were cultured at 37°C in Luria–Bertani (LB) media (Sambrook *et al.*, 1989). *S. cerevisiae* strains were cultured in YPAD at 30°C as described in Guthrie and Fink (1991), or grown in synthetic complete (SC) minus media for yeast two-hybrid analyses (James *et al.*, 1996). *M. xanthus* strains were grown in CTTYE media (CTT containing 0.2% yeast extract) at 33°C as previously described (Hodgkin and Kaiser, 1977).

Development, spore counts and β-galactosidase assays

Myxococcus xanthus development was induced on TPM agar as previously described (Kroos *et al.*, 1986). *M. xanthus* fruiting body morphology was assessed by light microscopy (Nikon SMZ800, Nikon Corporation, Tokyo, Japan). For assessing spore viability, *M. xanthus* cells induced to undergo development were harvested at 72 h post initiation and frozen at –80°C. Samples were then heat-treated at 50°C for 2 h, and then sonicated in a Sonifier 450 cup sonicator (Branson Corporation, Danbury, CT) at 5×, 50% power, for 5 min. Spore counts (Thöny-Meyer and Kaiser, 1993) and β-galactosidase enzymatic activity (Kroos *et al.*, 1986) were measured as previously described.

Cloning methods

In order to construct plasmid pDV456 for insertional disruption of *brgE*, an internal fragment of the gene was amplified by polymerase chain reaction (PCR) using primers BrgE-F and BrgE-R (Table 3). The amplicon, after digestion with EcoRI and PstI, was subsequently ligated into the vector pBGS18, which contains the *nptII* kanamycin-resistance (Km^R) gene. The resultant plasmid, pDV456, was introduced into *M. xanthus* cells by electroporation (Bio-Rad GenePulser Xcell), and Km^R transformants were screened by Southern blot for the desired disruption of the *brgE* ORF.

The GalK selection method (Ueki *et al.*, 1996) was used to attempt an in-frame deletion of the *birA* gene. Briefly, upstream primers BirA1 and BirA2 and downstream primers BirA3 and BirA4 were used to PCR-amplify regions flanking the *birA* ORF (Table 3). Both amplicons were ligated to the multiple cloning site of plasmid pBJ114 to form plasmid pDV757. DK1622 cells transformed with pDV757 as above were selected for kanamycin resistance and then screened for the presence of the deletion allele by growth on galactose and Southern blot.

To construct Gal4 BD fusions to full-length SdeK, or either its NTD or its CTD, the desired portions of the *sdeK* gene

Table 4. Strains and plasmids.

Strain/plasmid	Relevant characteristics and derivation	Reference
Strains		
E. coli		
JM109	*endA1 gyrA96 hsdR17 supE44 Δ(lac-proAB) recA1 relA1* F′[*traD36, proAB+*, $\lambda\alpha\chi\lambda\theta$, $\lambda\alpha\chi Z\Delta$M15]	Yanisch-Perron *et al.* (1985)
HB101	*F⁻ ara-14 galK2 hsdS20 (rB⁻,mB⁻) lacY1 leuB6 mtl-1 proA2 recA13 rpsL20 (strR) supE44 thi-1 xyl-5 λ⁻*	Boyer and Roulland-Dussoix (1969)
BL21	*dcm hsdS ompT gal/λ* (DE3)	Studier and Moffatt (1986)
M. xanthus[a]		
DK1622	Wild type	Kaiser (1979)
DK5511	Tn*5*-132*lac* Ω4414 (*devRS*)	Thöny-Meyer and Kaiser (1993)
DK5208	*csgA*::Tn*5-132*ΩLS205	Kroos and Kaiser (1987)
DK7160	Tn*5-132lac* Ω4400	Y. Cheng, Stanford University
DK7825	Tn5-132lac Ω4469 (*nsd*)	Gorski and Kaiser (1998)
DK7827	Tn*5-132lac* Ω4403	Gorski *et al*. (2000)
DK7862	Tn*5-132lac* Ω4408 (*sdeK*)	L. Gorski, Stanford University
VP664	*brgE*::pDV456	This work
VP825	*brgE*::pDV456 *csgA*::Tn*5-132*ΩLS205	This work
VP850	*brgE*::pDV456 Tn*5-132lac* Ω4403	This work
VP864	*brgE*::pDV456 Tn*5-132lac* Ω4469 (*nsd*)	This work
VP994	*brgE*::pDV456 Tn5-*132*lac Ω4414 (*devRS*)	This work
VP1006	*brgE*::pDV456 Tn5-*132*lac Ω4400	This work
VP1014	*brgE*::pDV456 Tn5-*132*lac Ω4408 (*sdeK*)	This work
S. cerevisiae		
PJ69-4A	*MATα trp1-901 leu2-3112 ura3-52 his3-200 gal4Δ gal80Δ LYS::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ*	James *et al.* (1996)
TII-1	Trp⁺, PJ69-4 A containing pDV310 ($SdeK_{CTD}$)	This work
TII-5	Trp⁺, PJ69-4 A containing pDV320 (full-length SdeK)	This work
TII-9	Trp⁺, PJ69-4 A containing pDV325 ($SdeK_{NTD}$)	This work
TII-158	Trp⁺, PJ69-4 A containing pGBD-C1	This work
TII-159	Trp⁺, Leu⁺, TII-1 containing pDV404	This work
TII-160	Trp⁺, Leu⁺, TII-5 containing pDV404	This work
TII-161	Trp⁺, Leu⁺, TII-9 containing pDV404	This work
TII-162	Trp⁺, Leu⁺, TII-158 containing pDV404	This work
Plasmids		
pGBD-C1	2 μ P_{ADH1}::*GAL4* BD *TRP1 bla* (Ap^R)	James *et al*. (1996)
pDV310	pGBD-C1 containing SdeK-F826/R1506 amplicon	This work
pDV320	pGBD-C1 containing SdeK-F19/R1506 amplicon	This work
pDV325	pGBD-C1 containing SdeK-F19/R852 amplicon	This work
pGAD-C1	2 μ P_{ADH1}::*GAL4* AD *LEU2 bla* (Ap^R)	James *et al*. (1996)
pDV404	pGAD-C1 containing *brgE*	This work
pBGS18	*nptII* (Km^R)	Spratt *et al*. (1986)
pDV456	pBGS18 containing BrgE-F/R amplicon	This work
pBJ114	Km^R, *galK*	Julien *et al*. (2000)
pDV757	pBJ114 containing BirA1/2 (upstream) and BirA3/4 (downstream) amplicons	This work
pGEX-KG	Carries glutathione S-transferase gene	Guan and Dixon (1991)
pGEX-6P-1	Cloning vector; carries glutathione S-transferase gene	Amersham Biosciences
pDV851	pGEX-6P-1 containing pDV404 insert	This work
pSG5-MycA	Cloning vector; carries T7 promoter and Kozak sequence	Jonas and Privalsky (2004)
pDV887	pSG5-MycA containing SdeK-F19/R1506 amplicon	This work

a. DK1622 is the parent wild-type strain for all *M. xanthus* strains in this study.

were amplified by PCR using the primers listed on Table 3. Primers SdeK-F19 and SdeK-R1506 were used to amplify a nearly full-length portion of *sdeK*, primers SdeK-F19 and SdeK-R852 were used to amplify the region encoding for the NTD, and primers SdeK-826 and SdeK-R1506 were used to amplify the region encoding for the CTD. Each amplicon was ligated, following digestion with appropriate restriction enzymes, into vector pGBD-C1 to produce plasmids pDV310 (CTD), pDV320 (full-length) and pDV325 (NTD). Plasmid pDV404 was obtained by yeast two-hybrid screen as described below. Plasmids pDV310, pDV320, pDV325 and pGBD-C1 were introduced into *S. cerevisiae* host strain PJ69-4A by the lithium acetate method (Gietz *et al*., 1997) to generate strains producing the SdeK C-terminal fusion (strain TII-1), the near full-length SdeK fusion (TII-5), the SdeK amino-terminal fusion (TII-9) and the vector control alone (TII-158) respectively.

All plasmids were introduced into *E. coli* strain JM109 using standard transformation methods (Sambrook *et al*., 1989), and maintained by culturing the strains in LB media supplemented with either kanamycin sulphate (40 μg ml^{-1}) or ampicillin (100 μg ml^{-1}).

Yeast two-hybrid assays

TII-5 cells were transformed with a yeast two-hybrid library

encoding for *M. xanthus* proteins fused to the Gal4 AD (Thomasson *et al*., 2002), and then plated onto SC Trp$^-$ Leu$^-$ His$^-$ (+ 1 mM 3-aminotriazole) and SC Trp$^-$ Leu$^-$ Ade$^-$ agar plates to test for complementation of the reporter auxotrophies, His$^-$ and Ade$^-$. Note that restoration of the Trp$^+$ and Leu$^+$ phenotypes was already provided by pDV320 (*trp1*$^+$) and the library plasmids (*leu2*$^+$) respectively. Of the approximately 100 000 transformants screened, only six had clones encoding for Gal4 AD fusion components having strong enough interactions with the SdeK–Gal4 BD fusion to allow for complementation of both of the reporter auxotrophies.

To identify the proteins interacting with SdeK, plasmid DNA was extracted (Zymo Research Zymoprep kit, Orange, CA) and introduced into *endA*$^+$ *E. coli* strain HB101. Transformants were plated onto M9 agar medium lacking leucine to select for maintenance of the *leu2*$^+$ library plasmid. Plasmid DNA was extracted from Leu$^+$ colonies by the alkaline lysis method (Sambrook *et al*., 1989), sent to the UC Davis DBS Sequencing Facility, and introduced into the *endA*$^-$ *E. coli* strain JM109 for stable maintenance of plasmids. Among the six plasmids identified by the screen, one, named pDV404, encoded for a BrgE-Gal4 AD fusion. The other five plasmids carried coding sequences for a hybrid response regulator (MXAN3879), a σ^{54}-dependent transcriptional regulator (MXAN5048), an ABC transporter permease (MXAN3773), a chorismate mutase/prephenate dehydratase (MXAN3221) and a DnaK homologue (MXAN5323); however, no *sdeK*-like developmental phenotypes were observed in mutants disrupted in any of these genes.

To determine with which of the SdeK constructs BrgE interacts, plasmid pDV404 was transformed as described above into strains TII-1, TII-5, TII-9 and TII-158 to generate strains TII-161, TII-160, TII-159 and TII-162 respectively. Strains TII-159 through 162 were streaked onto SC media lacking histidine or adenine to assess activation of the *HIS3* or *ADE2* reporter genes, respectively, as well as onto SC media containing X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) to assess activation of the *lacZ* reporter gene, as previously described (James *et al*., 1996; Pawlowski *et al*., 2003).

GST pulldown assay

The GST–BrgE bait fusion protein was constructed by inserting the coding sequence of plasmid pDV404, which contains the *brgE* gene, into GST cloning vector pGEX-6P-1 (Amersham Biosciences, Piscataway, NJ), to form plasmid pDV851. Exponential phase BL21 cells (Stratagene, La Jolla, CA) transformed with pDV851 or with pGEX-KG, a plasmid that can be induced to express a high quantity of GST, were grown in the presence of 1 mM Isopropyl-β-D-thiogalactopyranoside (IPTG) for 3 h at 37°C. The induced proteins were purified by incubation with pre-washed glutathione-Sepharose beads (Sigma-Aldrich, St. Louis, MO) and washed with TBS buffer (20 mM Tris, pH 7.6; 137 mM NaCl) containing complete protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN) at 4°C. To generate the SdeK prey protein, the SdeK-F19/R1506 amplicon was cloned into vector pSG5-MycA, to form plasmid pDV887. ^{35}S-methionine-labelled SdeK was generated from 2 μg of pDV887 by *in vitro* translation using the TNT kit (Promega Corporation, Madison, WI).

The GST pulldown assay was performed by incubating labelled SdeK with either glutathione-Sepharose-bound GST or GST–BrgE fusion proteins in TBS containing 10 mg ml^{-1} bovine serum albumin at 4°C for 1 h. After incubation, the beads were washed three times with 1 ml of TBS containing 0.1% Tween-20, and boiled in 1× SDS loading buffer (5% β-Mercaptoethanol). The eluted binding proteins were separated by a 10% SDS-polyacrylamide gel, and scanned with a Storm Imager 840 (Amersham Biosciences, Piscataway, NJ), and analysed using ImageQuant 5.2 software (Amersham Biosciences, Piscataway, NJ).

Predation assay

Assay conditions used were as reported previously (Pham *et al*., 2005). Briefly, an overnight culture of *Serratia marcescens* cells was inoculated onto a nitrocellulose membrane and allowed to form an even lawn of red-pigmented cells. Upon transfer to TPM starvation agar, wild-type and mutant *M. xanthus* cells were spotted separately onto the same lawn and allowed to swarm out by feeding on the *S. marcescens* cells. After 7–10 days, swarm sizes were imaged and quantified using the FluorChem 8900 imager (Alpha Innotech Corporation, San Leandro, CA). The predation phenotype of mutant strains is reported as a ratio of the swarm size of the mutant relative to the parent wild type.

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