

# The *asgE* locus is required for cell–cell signalling during *Myxococcus xanthus* development

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## Summary

In response to starvation, *Myxococcus xanthus* undergoes a multicellular developmental process that produces a dome-shaped fruiting body structure filled with differentiated cells called myxospores. Two insertion mutants that block the final stages of fruiting body morphogenesis and reduce sporulation efficiency were isolated and characterized. DNA sequence analysis revealed that the chromosomal insertions are located in open reading frames ORF2 and *asgE*, which are separated by 68 bp. The sporulation defect of cells carrying the *asgE* insertion can be rescued phenotypically when co-developed with wild-type cells, whereas the sporulation efficiency of cells carrying the ORF2 insertion was not improved when mixed with wild-type cells. Thus, the *asgE* insertion mutant appears to belong to a class of developmental mutants that are unable to produce cell–cell signals required for *M. xanthus* development, but they retain the ability to respond to them when they are provided by wild-type cells. Several lines of evidence indicate that *asgE* cells fail to produce normal levels of A-factor, a cell density signal. A-factor consists of a mixture of heat-stable amino acids and peptides, and at least two heat-labile extracellular proteases. The *asgE* mutant yielded about 10-fold less heat-labile A-factor and about twofold less heat-stable A-factor than wild-type cells, suggesting that the primary defect of *asgE* cells is in the production or release of heat-labile A-factor.

## Introduction

When *Myxococcus xanthus* is deprived of nutrients, ≈100 000 rod-shaped cells initiate a complex social interaction that culminates in the construction of a

multicellular structure called a fruiting body (Shimkets and Kaiser, 1982; Shimkets, 1990; Kaiser and Losick, 1993; Dworkin, 1996). After cells aggregate into fruiting bodies, individual rod-shaped cells within these structures begin to differentiate into spherical-shaped spores that are resistant to certain types of environmental stress. Thus, the *M. xanthus* development cycle occurs in a series of steps that include starvation, construction of a macroscopic fruiting body and differentiation of rod-shaped cells into spherical spores.

Co-ordinating multicellular development requires cell–cell communication, and a number of cell–cell signalling mutants have been isolated in previous analyses of the *M. xanthus* developmental cycle (Hagen *et al.*, 1978; LaRossa *et al.*, 1983; Downard *et al.*, 1993). These signalling mutants are unable to complete development by themselves, but they can overcome this developmental block when they are mixed with wild-type cells. Complementation does not involve a permanent genetic exchange from wild-type cells to mutant cells, as the mixed culture fruiting bodies contain spores that exhibit both wild-type and mutant phenotypes. Presumably, these *M. xanthus* mutants are defective for producing a cell–cell signal, but they retain the ability to respond to it. The results of complementation studies indicate that the signalling mutants can be placed into several different classes; mutants from the same class fail to complement each other, whereas mutants from different classes can rescue the developmental defects of their partner. This result led to the proposal that each class of mutant is defective for the production of a different cell–cell signal. The findings from subsequent studies are consistent with this proposal: each class of mutant is defective for the expression of particular groups of developmentally regulated *lacZ* reporter gene fusions (Kroos and Kaiser, 1984; 1987; Kroos *et al.*, 1986; Kuspa *et al.*, 1986).

Two groups of signalling mutants, the A-signal group and the C-signal group, have been well characterized. The defects observed for the A-signal group of mutants result from lesions in four genes, *asgA*, *asgB*, *asgC* (Kuspa and Kaiser, 1989), and *asgD* (Cho and Zusman, 1999). The *asg* mutants fail to produce normal levels of A-factor (or A-signal), which is proposed to be a mixture of amino acids and peptides generated by extracellular proteolysis (Kuspa *et al.*, 1992a; Plamann *et al.*, 1992). Work done by Kuspa *et al.* (1992b) suggests that the concentration of A-factor produced by developing cells

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may serve as a critical indicator of cell density. Hence, cells carrying an *asg* mutation are defective for aggregation, sporulation and full expression of developmentally regulated genes. Based on DNA sequence analyses of the *asg* genes, it was proposed that they encode components of a signal transduction pathway that regulates the expression of genes needed for A-signal production (Plamann *et al.*, 1994; 1995; Davis *et al.*, 1995).

The defects in aggregation and sporulation observed for the C-signal (also called C-factor) group are caused by mutations in a single locus called *csgA* (LaRossa *et al.*, 1983; Shimkets *et al.*, 1983; Shimkets and Asher, 1988). The fact that *csgA* mutants are defective for the expression of *lacZ* reporter gene fusions induced after 6 h of development, whereas *asg* mutants are defective for the expression of nearly all developmentally regulated *lacZ* fusions suggests that C-signal is needed later in development than A-signal (Kroos *et al.*, 1986; Kuspa *et al.*, 1986; Kroos and Kaiser, 1987). The product of the *csgA* gene is associated with the cell surface and has similarity to the short-chain alcohol dehydrogenase family of proteins (Kim and Kaiser, 1990a,b; 1991; Shimkets and Rafiee, 1990; Lee *et al.*, 1995). Aggregation and sporulation are triggered by different concentrations of CsgA; when the concentration of CsgA reaches a critical level, it can induce aggregation and, when it reaches a higher level, it can induce sporulation (Kim and Kaiser, 1991; Li *et al.*, 1992). This observation has led to the proposal that levels of C-signalling control two distinct morphological events, aggregation and sporulation, ensuring that they occur in the proper sequence. Consistent with this proposal is the finding that the cell–cell contacts that are likely to occur with increasing frequency as cells migrate to developing fruiting bodies promote the transmission of C-signal (Kim and Kaiser, 1990c,d).

A third class of cell–cell signalling mutants, called the E-signal group, was characterized in recent studies

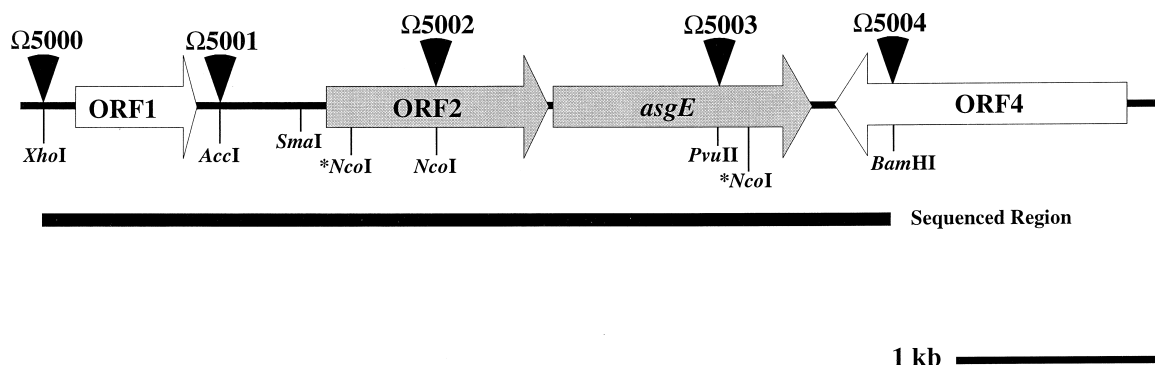
(Downard *et al.*, 1993; Downard and Toal, 1995; Toal *et al.*, 1995). The E-signal (or *esg*) mutants fail to aggregate or to sporulate normally, and they fail to express *lacZ* reporter gene fusions induced after 3–5 h of development, suggesting that E-signal may act somewhere between A-signal and C-signal. The finding that *esg* mutants can be complemented by wild-type cells on an agar surface, but not in suspension, suggests that transmission of E-signal may require cell–cell contacts. Biochemical analyses of *esg* mutants indicate that they are defective for the synthesis of branched-chain fatty acids, and that these compounds may be used for the production of E-signal.

In the work presented here, we characterized two insertion mutants that are unable to complete fruiting body development. DNA sequencing in the proximity of the insertions revealed that the insertions disrupted adjacent open reading frames designated ORF2 and *asgE*. As part of our initial characterization of these developmental mutants, we found that the sporulation defect of the *asgE* insertion mutant is alleviated when co-developed with wild-type cells, whereas the sporulation efficiency of the ORF2 insertion mutant is not improved when mixed with wild-type cells. Further analyses suggest that *asgE* developmental defects are the result of a reduced capacity to generate A-signal.

## Results

### *Developmental phenotypes caused by insertions in the asgE locus*

The *asgE* locus was originally defined by the  $\Omega$ 5002 and  $\Omega$ 5003 plasmid (pBMG3 and pREG-JP2B respectively) insertions in the chromosome of *M. xanthus* strain DK101 (see Table 1). The location of these insertions and the physical map derived from sequencing the surrounding



**Fig. 1.** Physical map of the *asgE* locus. The broadened black line indicates the 6.0 kb region of the *asgE* locus that was sequenced for this study. Boxes show the locations of the indicated open reading frames (ORFs), and the arrows show the predicted direction of transcription of these ORFs. Only those ORFs with the appropriate bias towards G + C nucleotides in the third position of codons are shown. Triangles mark the locations of the indicated plasmid insertions. ORF4 corresponds to ORF2 described previously by Harris and Singer (1998).

**Table 1.** Bacterial strains and plasmids.

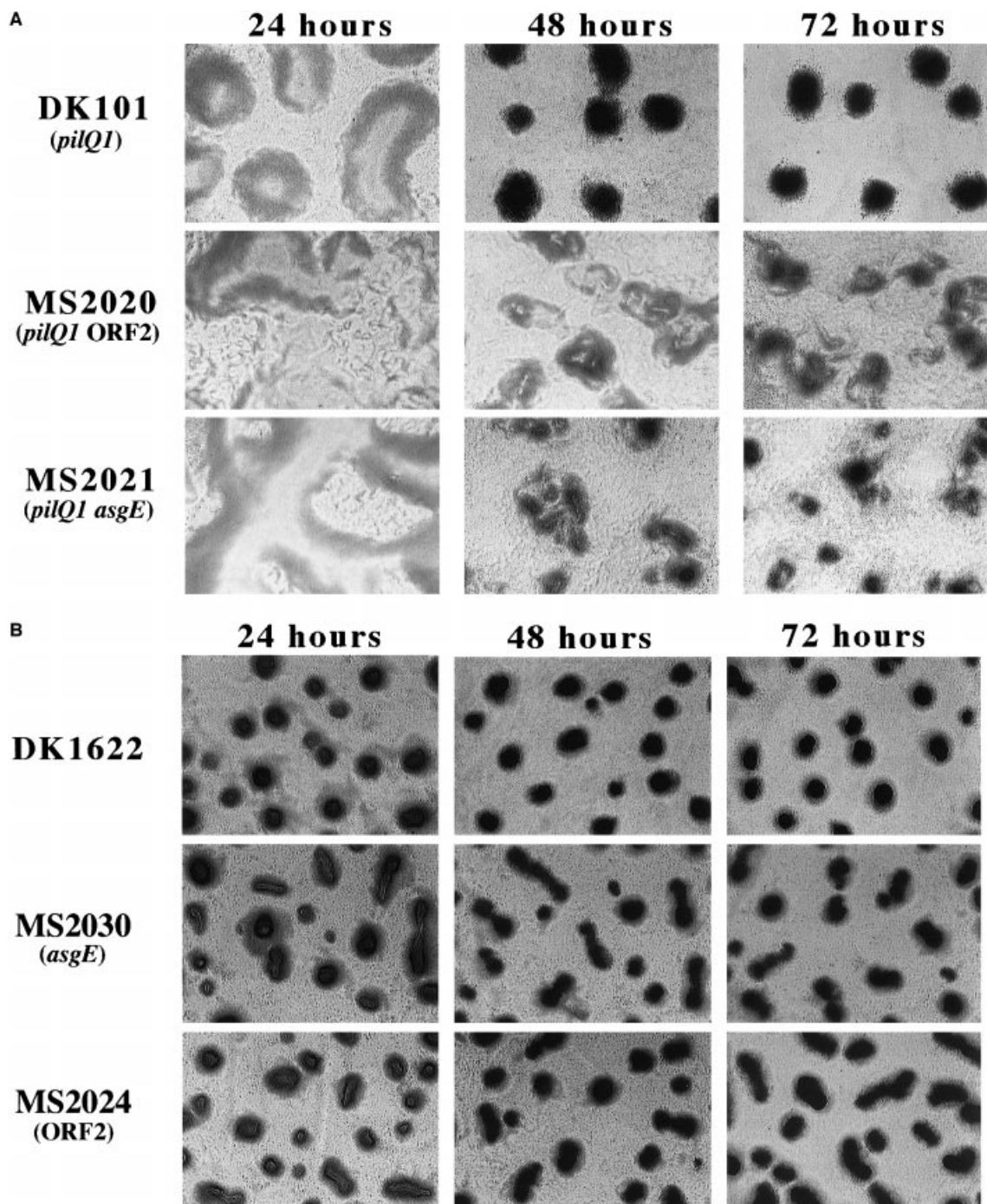
Strain or plasmid	Relevant characteristic	Source or reference
<i>E. coli</i> strains		
DH5 $\alpha$	<i>supE44</i> $\Delta$ <i>lacU169</i> $\phi$ 80 $\Delta$ <i>lacZ</i> M15 <i>hsdR17</i> <i>recA1 endA1 gyrA96 thi-1 relA1</i>	Messing <i>et al.</i> (1977)
JM101	$\Delta$ ( <i>lac pro</i> ) <i>thi supE F' traD36 proAB lac<sup>f</sup> <math>\Delta</math>lacZ</i> M15	Hanahan (1983)
<i>M. xanthus</i> strains		
DK101	<i>pilQ1</i> (wild-type development)	Hodgkin and Kaiser (1977)
DK476	<i>pilQ1 asgA476</i>	Hagen <i>et al.</i> (1978)
DK1622	Wild type	Kaiser (1979)
DK4323	<i>pilQ1 asgA476</i> $\Omega$ 4521Tn5 <i>lac</i>	Kuspa <i>et al.</i> (1986)
DK4398	<i>asgB480</i>	Kuspa and Kaiser (1989)
DK5216	<i>pilQ1 csgA::Tn5</i> $\Omega$ 205	Shimkets and Asher (1988)
DK5057	<i>asgA476</i>	Kuspa and Kaiser (1989)
JD275	<i>pilQ1 esg::Tn5</i> $\Omega$ 258	Downard <i>et al.</i> (1993)
MS2018	<i>pilQ1</i> pREG-JP5 ( $\Omega$ 5000)	This study
MS2019	<i>pilQ1</i> pAG101 ( $\Omega$ 5001)	This study
MS2020	<i>pilQ1</i> ORF2::pBMG3 ( $\Omega$ 5002)	This study
MS2021	<i>pilQ1 asgE::pREG-JP2B</i> ( $\Omega$ 5003)	This study
MS2022	<i>pilQ1</i> ORF4::pREG-JP3 ( $\Omega$ 5004)	This study
MS2024	ORF2::pBMG3 ( $\Omega$ 5002)	This study
MS2030	<i>asgE::pREG-JP2B</i> ( $\Omega$ 5003)	This study
Plasmids		
pBGS18	Kan <sup>r</sup>	Spratt <i>et al.</i> (1986)
pBluescript SKII	Amp <sup>r</sup>	Stratagene
pREG1727	Amp <sup>r</sup> Kan <sup>r</sup>	Fisseha <i>et al.</i> (1996)
pAG101	Kan <sup>r</sup> , pBGS18 containing 0.4 kb of DNA upstream of ORF2 on a <i>AccI</i> – <i>SmaI</i> fragment	This study
pBMG3	Kan <sup>r</sup> , pBGS18 containing 0.5 kb of ORF2 on a <i>XbaI</i> – <i>Bam</i> HI fragment	This study
pELF3	Kan <sup>r</sup> , pBGS18 containing ORF2, <i>asgE</i> , and 0.4 kb of downstream DNA on a <i>Bam</i> HI– <i>Pst</i> I fragment	This study
pREG-JP2B	Amp <sup>r</sup> , Kan <sup>r</sup> , pREG1727 containing 1.0 kb of <i>asgE</i> on a <i>Bam</i> HI– <i>Hind</i> III fragment	This study
pREG-JP3	Amp <sup>r</sup> , Kan <sup>r</sup> , pREG1727 containing 0.4 kb of <i>asgE</i> and 0.4 kb of downstream DNA on a <i>Bam</i> HI– <i>Hind</i> III fragment	This study
pREG-JP5	Amp <sup>r</sup> , Kan <sup>r</sup> , pREG1727 containing 1.3 kb of DNA upstream of ORF2 on a <i>Bam</i> HI– <i>Hind</i> III fragment	This study

region are shown in Fig. 1 (GenBank accession number AF060075). The 3' end of this newly sequenced region of DNA overlaps with the DNA sequence described previously by Harris and Singer (1998). Open reading frame 4 (ORF4) in Fig. 1 corresponds to ORF2 in Harris and Singer (1998). The  $\Omega$ 5002 insertion is within a 1.0 kb open reading frame designated ORF2. The second plasmid insertion,  $\Omega$ 5003, occurred in a 1.4 kb ORF (designated *asgE*), which is located 68 bp downstream of ORF2 and is predicted to be transcribed in the same orientation as ORF2. Each of these chromosomal insertions is expected to produce two incomplete copies of the target gene.

To determine whether cells carrying the  $\Omega$ 5002 insertion in ORF2 (MS2020) or the  $\Omega$ 5003 insertion in *asgE* (MS2021) form fruiting bodies, we observed the progress of their developmental cycle and compared it with that of parental DK101 cells (Fig. 2A). Cells of strain DK101 show extensive aggregation after 24 h of development on TPM starvation agar, and these aggregates of cells compact into darkened fruiting body structures after 48 h. In contrast, MS2020 and MS2021 cells aggregate,

but these aggregates of cells fail to compact into normal-looking fruiting structures after 72 h of development on TPM agar. No obvious changes in the morphology of MS2020 and MS2021 fruiting bodies were observed after this 72 h period.

DK101, which is the parental strain of MS2020 and MS2021, carries the social motility mutation *pilQ1* (formerly *sglA1*) (Hodgkin and Kaiser, 1977), and previous work has shown that the *pilQ1* mutation enhances the defects of certain developmental mutants (Cheng and Kaiser, 1989; Kuspa and Kaiser, 1989). To determine whether insertions in the *asgE* locus produce developmental defects in an otherwise wild-type background, the  $\Omega$ 5002 and  $\Omega$ 5003 insertions were transduced into *pilQ1*<sup>+</sup> strain DK1622 using phage Mx8. After identifying transductants carrying the *pilQ1*<sup>+</sup> allele and the  $\Omega$ 5002 or  $\Omega$ 5003 insertion, we spotted cells onto TPM agar plates and monitored their development (Fig. 2B). Fruiting bodies were observed for cells carrying the  $\Omega$ 5002 insertion in ORF2 (MS2024) and for cells carrying the  $\Omega$ 5003 insertion in *asgE* (MS2030). However, the fruiting



**Fig. 2.** Behaviour of *M. xanthus* cells during development on TPM agar plates. Cells were spotted on TPM starvation agar and monitored visually as described in *Experimental procedures*. Development of the indicated strains was observed for 3 days, and photographs were taken after 24, 48 and 72 h using a total magnification of 40 $\times$ .

A. Mutations in the DK101(*pilQ1*) background.

B. Mutations in the DK1622 background.

bodies formed by these insertion mutants are less compact than wild-type DK1622 fruiting bodies. In addition, MS2024 and MS2030 fruiting bodies are often elongated compared with wild-type fruiting bodies, which are typically round. Thus, the aggregation defect produced

by the  $\Omega$ 5002 and  $\Omega$ 5003 insertions appears to be less pronounced in the *pilQ*<sup>+</sup> background of strain DK1622 than in the *pilQ1* background of strain DK101, but it can still be detected using our standard assay conditions.

The sporulation efficiencies of the MS2020 and MS2021

**Table 2.** Developmental phenotypes of wild-type and mutant strains.

Strain	Fruiting body development <sup>a</sup>	Percentage wild-type sporulation <sup>b</sup>
DK101 ( <i>pilQ1</i> )	+	100.0 ± (23.9)
MS2018 ( <i>pilQ1</i> Ω5000)	+	45.0 ± (16.6)
MS2019 ( <i>pilQ1</i> Ω5001)	+	63.9 ± (10.1)
MS2020 ( <i>pilQ1</i> ORF2::Ω5002)	-	0.6 ± (0.2)
MS2021 ( <i>pilQ1 asgE</i> ::Ω5003)	-	1.1 ± (0.7)
MS2022 ( <i>pilQ1</i> ORF4::Ω5004)	+	83.0 ± (20.9)
DK1622	+	100.0 ± (33.0)
MS2024 (ORF2::Ω5002)	±	35.4 ± (4.4)
MS2030 ( <i>asgE</i> ::Ω5003)	±	3.7 ± (2.0)

a. The + sign means fruiting bodies were detected visually, and the - sign means fruiting bodies were not detected visually. The ± sign indicates that fruiting bodies were detected, but they appeared to be less compacted than wild-type fruiting bodies.

b. Values were determined by transferring heat/sonication-resistant spores to CTT agar plates, incubating the plates for 5 days and counting the number of colonies that arose from germinated spores. Cells were placed on TPM agar and allowed to develop for 5 days. Development was monitored visually as described in *Experimental procedures*. Spore viability assays were used to determine the sporulation efficiency of each strain. The mean values for the spore assays were determined from three independent experiments. Mean values are given as a percentage of parental strain DK101 or DK1622, and standard deviations are shown in parentheses.

mutants, as well as their *pilQ*<sup>+</sup> counterparts, were examined after 5 days on TPM starvation agar, and compared with parental strains DK101 and DK1622 (Table 2). For MS2021 cells that carry the *pilQ1* allele and Ω5003 insertion in *asgE*, a 100-fold reduction in viable spore numbers was observed compared with cells from parental strain DK101. The sporulation defect of MS2020 cells that carry the *pilQ1* mutation and the Ω5002 insertion in ORF2 was found to be roughly the same as MS2021 cells; MS2020 cells yielded 160-fold less viable spores than DK101 cells. When the number of viable spores produced by MS2030 cells (Ω5003 *pilQ*<sup>+</sup>) was compared with MS2021 cells (Ω5003 *pilQ1*), we found that they had similar reductions in sporulation efficiency. In contrast, MS2024 cells that carry the ORF2 insertion and a wild-type copy of *pilQ* show only a small reduction in sporulation efficiency (threefold) compared with MS2020 cells that carry this insertion and the *pilQ1* mutation (160-fold). Apparently, the *pilQ1* allele enhances the sporulation defect of cells carrying the Ω5002 insertion in ORF2, but this allele does not contribute to the sporulation deficiency of cells carrying the Ω5003 insertion in *asgE*.

#### *Localizing the region of the asgE locus that is required for development*

To help delimit the region of the *asgE* locus that is required for development, we made plasmid insertions flanking ORF2 and *asgE* (Fig. 1). One of these plasmid

insertions, designated Ω5004, is located 400 bp downstream of *asgE*. When cells carrying this insertion were spotted onto TPM agar plates, they formed normal-looking fruiting bodies and sporulated at levels similar to that of parental DK101 cells (Table 2). Two plasmid insertions were made in the region upstream of ORF2. The first insertion, called Ω5000, is located ≈1.4 kb upstream of ORF2. The second insertion, Ω5001, was made 509 bp upstream of ORF2. When spotted onto TPM agar plates, cells carrying the Ω5000 insertion or the Ω5001 insertion produced normal-looking fruiting bodies, and the number of viable spores produced by these fruiting bodies was about half that of DK101 fruiting bodies (Table 2). Hence, the Ω5000, Ω5001 and Ω5004 insertions led to relatively mild developmental defects compared with those produced by the original insertions in the *asgE* locus. Based on these results, we propose that the region of the *asgE* locus that is critical for fruiting body morphogenesis is within the 3.2 kb of DNA that separates Ω5001 and Ω5004 (extending from 509 bp upstream of ORF2 to 400 bp downstream of *asgE*).

When the results of our insertion mutagenesis are examined in the context of the physical map shown in Fig. 1, it is clear that the Ω5004 insertion falls within a 1.5 kb ORF that is located immediately downstream of *asgE*, in what appears to be a different operon. Our data show that cells carrying the Ω5004 insertion at the 3' end of ORF4 develop normally. This result is consistent with the findings of a previous study by Harris and Singer (1998); they also reported that an insertion in this ORF had no effect on development. Taken together, these data suggest that the developmental defects caused by the Ω5002 and Ω5003 insertions are not simply the result of a polar effect on a gene downstream of *asgE*. Moreover, these findings imply that the most likely explanation for the Ω5003 phenotype is an inactivation of *asgE* function.

#### *DNA sequence analysis of asgE*

To characterize the *asgE* locus further, we determined the DNA sequence for a 6 kb region extending from the *Bam*HI site to the *Xho*I site. The four ORFs that have a strong bias towards G + C nucleotides in the third position of codons (91.3% for ORF1, 90.1% for ORF2, 91.8% for *asgE* and 92.2% for ORF4) are shown in Fig. 1. This bias is typical for protein coding regions in *M. xanthus* and other high-G + C organisms (Bibb *et al.*, 1984; Oyaizu and Woese, 1985), indicating that we have probably identified *bona fide* ORFs.

When we used the ORFs identified in the *asgE* locus to search the GenBank database, similarities were found to the product of *asgE*, but not to the other three ORFs. *AsgE* has similarity to proteins from a variety of eukaryotic and prokaryotic organisms, including *C. elegans*, *S. pombe*,

*M. thermoaerophilum* and *Pseudomonas* strain ADP. These similarities span the entire length of the AsgE protein, which is predicted to be 462 amino acids. AsgE has the highest degree of similarity (55%) and identity (38%) to an uncharacterized ORF (F38E11.3) from *C. elegans* (Wilson *et al.*, 1994). Among the proteins that have a known function, AsgE has the highest similarity (40%–45%) and identity (20%–23%) to AtzA and AtzB from *Pseudomonas* strain ADP (De Souza *et al.*, 1996; Boundy-Mills *et al.*, 1997). Biochemical analysis has shown that AtzA and AtzB are enzymes that allow *Pseudomonas* strain ADP to degrade the herbicide atrazine (De Souza *et al.*, 1996; Boundy-Mills *et al.*, 1997). Recent work by Sadowsky *et al.* (1998) suggests that AtzA and AtzB belong to a ubiquitous family of amidohydrolase proteins that catalyse a variety of hydrolytic reactions, often using substrates that contain nitrogen–carbon heterocyclic rings. Further analysis of the AsgE protein sequence revealed two potential membrane-spanning domains. These hydrophobic domains span residues 261–280 in the middle of the AsgE protein and residues 404–421 in the C-terminus of AsgE.

#### Extracellular complementation of mutants by wild-type cells

The sporulation defect of some developmental mutants can be rescued phenotypically when they are mixed with wild-type cells, a phenomenon that has been referred to as extracellular complementation (Hagen *et al.*, 1978; LaRossa *et al.*, 1983; Downard *et al.*, 1993). These mutants are defective for production of cell–cell signals that are required for multicellular development, but they retain the ability to respond to these signals when they are co-cultured with wild-type cells. As part of our initial characterization of the ORF2 and *asgE* insertion mutants, we tested their ability to be complemented extracellularly by mixing them with wild-type cells. The sporulation efficiencies of these mutants after 5 days on TPM

starvation agar are shown in Table 3. When cells carrying the *asgE* insertion (MS2021) were mixed with DK101 cells in a 1:1 ratio, we found that the sporulation defect caused by the insertion in *asgE* was rescued completely. In fact, the results presented in Table 3 suggest that the absolute level of sporulation for the *asgE* insertion mutant in the presence of wild-type cells is higher than that of wild-type cells alone. Similar results were obtained in a previous analysis of *asg* mutants, which are defective for the production of A-signal (Kuspa and Kaiser, 1989). In this mixing experiment, the MS2021 spores produced colonies that retained their developmental defects, indicating that a permanent genetic exchange did not take place between wild-type and MS2021 cells. Thus, the *asgE* insertion mutant shares at least one property with *M. xanthus* cell–cell signalling mutants: it can be complemented extracellularly. In contrast to MS2021 cells, MS2020 cells, which carry the ORF2 insertion, showed no improvement in sporulation efficiency when they were mixed with wild-type cells in a 1:1 ratio. Consistent with this finding is the fact that the sporulation defect of cells carrying a deletion of both ORF2 and *asgE* could not be rescued when co-developed with wild-type cells (data not shown). Apparently, cells carrying the ORF2 insertion differ from those carrying an *asgE* insertion with regard to their ability to be complemented extracellularly.

#### Extracellular complementation by *asgA*, *csgA* and *esg* cells

Three cell–cell signals that have been well characterized are A-signal, C-signal and E-signal. *M. xanthus* mutants that are defective for the production of these signals have a variety of developmental defects, including an arrest in aggregation and a reduction in sporulation efficiency (LaRossa *et al.*, 1983; Shimkets *et al.*, 1983; Shimkets and Asher, 1988; Kuspa and Kaiser, 1989; Downard *et al.*, 1993). Previous studies have shown that mutants that are defective for production of the same cell–cell signal

**Table 3.** Sporulation of mutants in cell mixtures.

Strain mixture		Percentage wild-type sporulation
Test strain	Co-culture strain	Test strain
MS2020 ( <i>pilQ1</i> ORF2:Ω5002)	DK101 ( <i>pilQ1</i> )	0.8 ± (0.5)
MS2021 ( <i>pilQ1 asgE</i> ::Ω5003)	DK101 ( <i>pilQ1</i> )	326.4 ± (65.5)
MS2021 ( <i>pilQ1 asgE</i> ::Ω5003)	DK476 ( <i>pilQ1 asgA476</i> )	0.8 ± (0.2)
MS2021 ( <i>pilQ1 asgE</i> ::Ω5003)	DK5216 ( <i>pilQ1 csgA</i> )	169.4 ± (14.7)
MS2021 ( <i>pilQ1 asgE</i> ::Ω5003)	JD275 ( <i>pilQ1 esg</i> )	83.3 ± (12.2)

The indicated strains were mixed in a 1:1 ratio and spotted onto TPM agar plates as described in *Experimental procedures*. The sporulation efficiencies of test strains were determined after 5 days of development as described in Table 2. The mean values for the spore assays were determined from three independent experiments and normalized to the mean value for strain DK101 alone. Standard deviations are shown in parentheses next to the means.

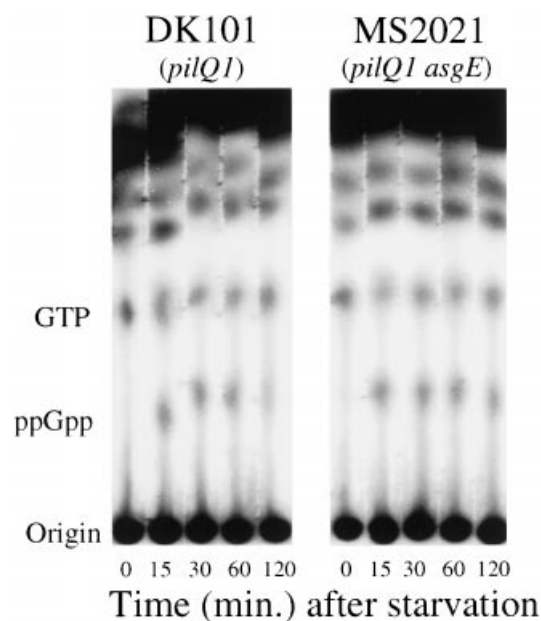
fail to complement one another when they are co-developed. To examine whether the *asgE* insertion mutant is defective for the production of one of these three cell–cell signals, we co-developed strain MS2021 with cells that are defective for the production of A-signal, C-signal or E-signal (*asgA*, *csgA* and *esg* cells respectively). The sporulation efficiency of MS2021 cells after 5 days of development is shown in Table 3. When co-developed with *csgA* or *esg* cells, MS2021 cells sporulate at or near wild-type levels, suggesting that *csgA* and *esg* cells can provide the signal that MS2021 cells fail to produce. In contrast, the sporulation deficiency of MS2021 cells was not improved when co-developed with *asgA* cells (Tables 2 and 3), indicating that MS2021 cells may be defective for the production of the extracellular A-signal. When we performed the reciprocal experiments, we found that MS2021 cells rescued the sporulation defect of *csgA* and *esg* cells (data not shown). However, MS2021 cells failed to rescue the sporulation defect of *asgA* cells fully; the sporulation efficiency of *asgA* cells increased from < 0.001% to about 65.0% when co-developed with wild-type cells, but it increased to only 4.0% when co-developed with MS2021 cells, the same sporulation efficiency seen in *asgE* cells.

#### Starvation recognition in *asgE* cells

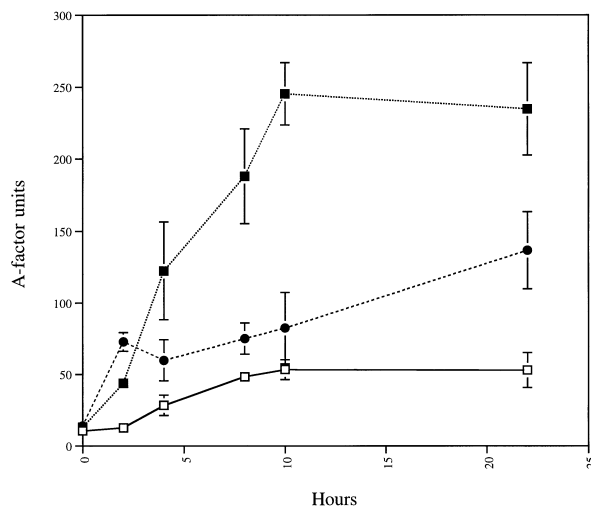
Previous work by Harris *et al.* (1998) showed that mutants defective for synthesis of the intracellular signalling molecule (p)ppGpp failed to produce normal levels of A-factor. Presumably, these mutants fail to initiate the intracellular starvation response that precedes A-signal production, rather than being defective in the A-signal production pathway itself. To examine whether the *asgE* mutant is defective for (p)ppGpp synthesis, we compared the levels of (p)ppGpp in MS2021 cells with the levels found in wild-type cells. The results in Fig. 3 show that the levels of (p)ppGpp in MS2021 cells are similar to those of wild-type DK101 cells. Hence, the *asgE* mutant does not appear to be defective for (p)ppGpp synthesis. These data strongly suggest that the developmental defects of *asgE* cells are caused by lack of A-signal rather than starvation recognition.

#### A-factor production in the *asgE* insertion mutant

To examine the ability of the *asgE* insertion mutant to produce extracellular A-signal (or A-factor) directly, we performed A-factor assays in cell suspension. Conditioned medium from DK101 (*asgE*<sup>+</sup>, *asgA*<sup>+</sup>), DK476 (*asgE*<sup>+</sup>, *asgA*) or MS2021 (*asgE*, *asgA*<sup>+</sup>) served as the source of A-factor, and A-factor activity was monitored using the A-factor-dependent *lacZ* reporter gene fusion  $\Omega$ 4521 as described previously (Plamann *et al.*, 1992).



**Fig. 3.** Synthesis of (p)ppGpp in wild-type and *asgE* cells. One-dimensional thin-layer chromatographs of cell extracts of strains DK101 and MS2021. Each lane was loaded with an equal number of counts at the origin. The migration locations of ppGpp and GTP are designated. Extracts were made from cells at the indicated times after transfer to starvation medium.



**Fig. 4.** A-factor production in mutant and wild-type cells. A-factor activity was assayed by determining the amount of  $\beta$ -galactosidase produced by the tester strain DK4323. DK4323 carries the A-factor-dependent *lacZ* reporter gene fusion  $\Omega$ 4521 and the *asgA476* allele, which renders this strain deficient for A-signalling. The source of A-factor for DK4323 was cell-free MC7 buffer conditioned by donor strain DK101 (black squares), DK476 (white squares) or MS2021 (black circles). Assays were performed as described previously by Plamann *et al.* (1992). The values shown are means derived from three independent experiments. Error bars are standard deviations of the means.

The results shown in Fig. 4 indicate that the levels of A-factor produced by *asgE* cells are about 30.0% of wild-type cells after 10 h and about 50.0% of wild-type cells after 22 h. Compared with *asgA* cells, which are known to be defective for the production of A-factor, the level of A-factor produced by *asgE* cells is 1.5- to 2.5-fold higher. Thus, the levels of A-factor produced by *asgE* cells fall between those of wild-type cells and *asgA* cells.

#### Heat-labile and heat-stable A-factor activity in *asgE* cells

In conditioned medium, two substances have been shown to have A-factor activity (Plamann *et al.*, 1992; Kuspa *et al.*, 1992a). The first substance is heat stable and consists of a mixture of amino acids and peptides. The second substance is heat labile and contains at least two proteases that are released into the extracellular media. To determine whether *asgE* cells are defective for one or both of these substances, we compared heat-stable and heat-labile activity in medium conditioned by MS2021 cells with that of wild-type cells (Table 4). Compared with wild-type cells, MS2021 cells produce about 10-fold less heat-labile A-factor. In contrast, the heat-stable A-factor activity in *asgE* cells is reduced only about twofold compared with wild-type cells. Taken together, these findings suggest that the primary defect in *asgE* cells is the production or release of heat-labile A-factor.

#### Phenotypic rescue of the *asgE* mutant by increasing cell density and by pronase

In their analysis of *asg* mutants, Kuspa *et al.* (1992b) also showed that the sporulation deficiency of an *asgB* mutant, which produces A-factor at about 5.0–10.0% of wild-type levels, can be rescued by increasing the cell density during development by 10- to 20-fold. This finding can be explained by the fact that A-factor is produced in proportion to cell density. Based on these results, we predicted that the sporulation defect of the *asgE* insertion mutant, which produces A-signal at about 30–50% of wild-type levels, could be rescued by increasing its density during development by about two- to threefold. Accordingly, we spotted MS2021 cells onto TPM agar plates at various cell densities and examined their sporulation efficiencies (Fig. 5). Little change was observed in the sporulation efficiency of wild-type cells after increasing their density twofold. However, increasing the density of MS2021 cells twofold improved their sporulation efficiency  $\approx$ 12-fold, to about 50.0% of wild-type levels. When the density of MS2021 cells was increased threefold, no additional improvement in sporulation efficiency was observed (data not shown). However, it should be noted that the sporulation efficiency of wild-type cells was

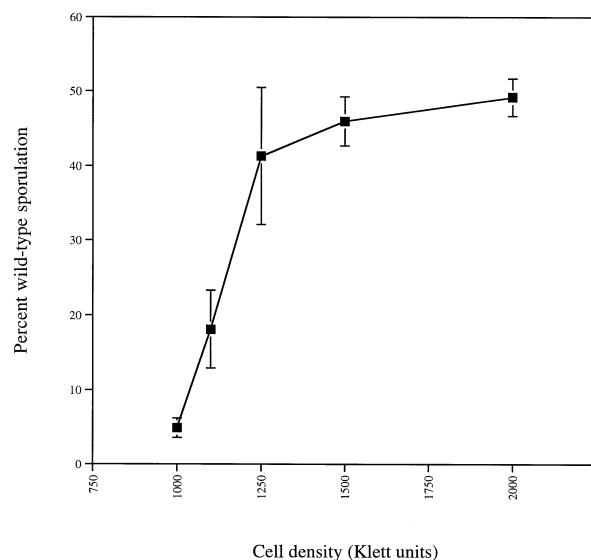
**Table 4.** Heat-stable and heat-labile A-factor activity.

Strains	A-factor activity	
	Heat stable	Heat labile
DK101 ( <i>pilQ1</i> )	232 $\pm$ (9)	118 $\pm$ (8)
MS2021 ( <i>pilQ1 asgE::<math>\Omega</math>5003</i> )	123 $\pm$ (35)	11 $\pm$ (3)

A-factor activity was measured using tester strain DK4323. The source of A-factor for DK4323 was cell-free MC7 buffer conditioned by donor strain DK101 or MS2021. Heat-stable and heat-labile A-factor were isolated and assayed as described previously by Plamann *et al.* (1992). The values shown are means derived from three independent experiments. Standard deviations are shown in parentheses next to the means.

somewhat lower after increasing their density threefold during development.

Because *asgE* cells appear to be lacking the heat-labile component of A-factor, we assayed whether the addition of an exogenously added protease could rescue the *asgE* phenotype. Previous studies have shown that the mixture of proteases in pronase have A-factor activity and that the sporulation defect of *asgB* mutants can be rescued when pronase is provided to them during development in submerged culture (Plamann *et al.*, 1992; Kuspa *et al.*, 1992b). Presumably, the proteases in pronase are able to generate A-factor by extracellular proteolysis of proteins from *asg* cells, which in turn allows them to complete development. To test whether the sporulation defect of



**Fig. 5.** Rescue of sporulation in the *asgE* insertion mutant by increasing cell density. MS2021 cells were spotted onto TPM agar plates at the cell densities shown and allowed to develop for 5 days. The black squares show the sporulation efficiency of MS2021 cells at each cell density. Sporulation efficiencies were determined by comparing the number of viable spores produced by MS2021 cells at the indicated cell density with the number of viable spores produced by wild-type DK101 cells at the same cell density. The values shown are means derived from three independent experiments, and the error bars are standard deviations of the means.

**Table 5.** Rescue of sporulation by pronase.

Strains	Percentage wild-type sporulation	
	- Pronase	+ Pronase
DK1622	100.0 ± (34.8)	100.0 ± (10.9)
DK4398 ( <i>asgB480</i> )	0.2 ± (0.1)	194.9 ± (11.8)
DK5057 ( <i>asgA476</i> )	< 0.005	< 0.005
MS2030 ( <i>asgE::Ω5003</i> )	11.8 ± (0.6)	2.3 ± (1.1)

Cells of wild-type and mutant strains were placed in submerged culture and allowed to develop for 5 days. Pronase was added to the cultures at 40 µg ml<sup>-1</sup> as indicated. The sporulation efficiency of each strain was determined as described in Table 2. Mean values were derived from three independent experiments and are presented as a percentage of parental strain DK1622. Standard deviations are shown in parentheses next to the means.

the *asgE* insertion mutant can be rescued by the addition of pronase, we placed MS2030 cells in submerged culture wells containing MC7 buffer, then added 40 µg ml<sup>-1</sup> pronase to half of the culture wells. Table 5 shows the sporulation efficiency of MS2030 cells after development in submerged culture for 5 days. In the absence of pronase, the sporulation efficiency of MS2030 cells is about 12.0% that of wild-type DK1622 cells. When MS2030 cells developed in the presence of pronase, the sporulation efficiency was lower, dropping to about 2.0% of wild-type cells. For comparison, we examined whether the sporulation deficiency of two A-signalling-defective mutants could be rescued by the addition of pronase (Table 5). Consistent with the findings of Kuspa *et al.* (1992a), we observed that the sporulation defect of the *asgA* mutant was not improved by adding pronase, whereas the sporulation deficiency of the *asgB* mutant was completely rescued in the presence of pronase. Thus, like *asgA* cells, it appears that cells carrying the Ω5003 insertion in *asgE* cannot be rescued by pronase, implying that the A-signalling defect in *asgE* cells is not simply the result of a lack of protease production.

## Discussion

### *Insertions in the asgE locus inhibit M. xanthus development*

The *asgE* locus was originally defined by the Ω5002 and Ω5003 insertions in the *M. xanthus* chromosome. DNA sequence surrounding Ω5002 and Ω5003 revealed that these insertions occurred in different but closely linked ORFs, which we designated ORF2 and *asgE* respectively. The ORF2 and *asgE* genes are separated by only 68 bp and are predicted to be transcribed in the same direction. Hence, these two genes may be part of the same transcriptional unit. Whether or not this is the case, *asgE* is likely to be the last gene in an operon, because an ORF that is predicted to be transcribed in the opposite

direction is located ≈100 bp downstream of the *AsgE* protein coding region. Based on this fact and the finding that an insertion located only 400 bp downstream of *asgE* has no effect on development, we believe that the defects observed for cells carrying Ω5003 are caused by disruption of the *asgE* gene and not by a polar effect on a downstream gene. For cells carrying the Ω5002 insertion in ORF2, the cause of the observed developmental defects is less clear because Ω5002 has the potential to exert a polar effect on transcription of the downstream *asgE* gene. However, the fact that the ORF2 insertion mutant cannot be complemented extracellularly, whereas the *asgE* insertion mutant can be complemented in mixtures with wild-type cells, argues that the defects caused by Ω5002 are not simply a result of a polar effect on *asgE*.

In an otherwise wild-type background, the Ω5003 insertion in *asgE* produces defects in two critical events in the *M. xanthus* developmental cycle: the final compacting of cell aggregates into well-defined, symmetrical mounds and sporulation. Thus, the *asgE* mutant appears to be unable to complete the final stages of fruiting body development. Cells carrying the Ω5002 insertion in ORF2 seem to be blocked at roughly the same point in the process of constructing macroscopic fruiting body structure. However, compared with the Ω5003 insertion in *asgE*, the Ω5002 in ORF2 produces a relatively mild defect in sporulation in an otherwise wild-type background; the sporulation efficiency of cells carrying the Ω5002 insertion is down about threefold compared with wild-type cells, whereas the sporulation efficiency of cells carrying the Ω5003 insertion is down about 30-fold. In the *pilQ1* background in which the Ω5002 insertion was first isolated, the defect in sporulation is more severe; the sporulation efficiency of cells carrying Ω5002 is down about 160-fold compared with wild-type cells. In contrast, the sporulation efficiency of cells carrying the Ω5003 insertion in *asgE* is similar in a *pilQ1* and a *pilQ*<sup>+</sup> background. These results suggest that there is a synergistic relationship between *pilQ1* and the mutation caused by the Ω5002 insertion, an effect that has been observed in previous studies of *M. xanthus* developmental mutants (Cheng and Kaiser, 1989; Kuspa and Kaiser, 1989).

### *Cells carrying the asgE insertion are defective for production of A-signal*

Our preliminary characterization of the *asgE* insertion mutant showed that its sporulation defect can be corrected when co-developed with wild-type cells, suggesting that the *asgE* insertion mutant is defective for cell-cell signalling. Based on the following results, we believe that *asgE* cells fail to produce normal levels of the extracellular A-signal: (i) an *asgA* mutant, which is known

to be defective for A-signalling, fails to rescue the sporulation deficiency of *asgE* cells; (ii) bioassays that use expression of the A-signal-dependent *lacZ* reporter gene fusion  $\Omega 4521$  to measure A-factor production indicate that the levels of A-factor produced by *asgE* cells are about 30.0–50.0% of those of wild-type cells; and (iii) when the density of *asgE* cells was raised twofold above the standard level during development, the sporulation efficiency of the *asgE* cells was raised from about 3.0% of wild-type cells to about 50.0% of wild-type cells. In view of these results and the finding that *asgE* cells produce about 1.5- to 2.5-fold higher levels of A-factor than *asgA* cells, one could imagine that the developmental defects of the *asgE* insertion mutant would be less severe than the original collection of A-signalling-defective mutants isolated by Hagen *et al.* (1978).

A-signal is proposed to be a mixture of amino acids and peptides that is generated by extracellular proteolysis (Kuspa *et al.*, 1992a; Plamann *et al.*, 1992). This cell–cell signal is thought to function as an indicator of cell density, similar to the way in which homoserine lactones function as quorum-sensing signals (Kuspa *et al.*, 1992b; Dworkin, 1996). Thus, by sampling the concentration of A-signal, *M. xanthus* ensures that a sufficient number of cells are present to complete fruiting body development.

The original collection of mutations that inhibit the production of A-signal were mapped to three genes, *asgA*, *asgB* or *asgC* (Kuspa and Kaiser, 1989). The DNA sequence of the *asgA* and *asgB* genes suggests that they encode components of a signal transduction pathway (Plamann *et al.*, 1994; 1995). For example, AsgA may function as an intermediate in a phosphorelay system, such as the one that controls the initiation of sporulation in *Bacillus subtilis* (Burbulys *et al.*, 1991), and AsgB appears to be a DNA-binding protein, suggesting that it could function as a transcription factor at the end of the phosphorelay system. It seems that a likely role for these proteins in the A-signalling pathway is to alter gene expression in response to some input signal, which in turn leads to the production of A-factor, rather than being directly involved in the synthesis of the A-signal itself. Recently, Cho and Zusman (1999) identified a new *asg* locus, which they called *asgD*. It was proposed that AsgD is involved in sensing nutritionally limiting conditions, presumably through its role in the A-signal phosphorelay system.

How could AsgE fit into the A-signalling pathway? When co-developed with wild-type cells, the *asgA* and *asgE* mutants sporulate at or near wild-type levels. In mixing experiments with *asgA* and *asgE* mutants, we found that the *asgE* cells improved the sporulation efficiency of *asgA* cells to a level that is roughly the same as that observed for a pure culture of *asgE* cells. However, this relationship is not reciprocal; when *asgE*

cells are co-developed with an *asgA* mutant, the *asgE* cells showed no improvement in sporulation efficiency. Our interpretation of this finding is that AsgE functions downstream of AsgA in the A-signal production pathway. Hence, it is tempting to speculate that the *asgE* gene may be one of the targets of the AsgA signalling system proposed by Plamann *et al.* (1994; 1995).

The *asgE* mutant is almost completely deficient in heat-labile A-factor activity, yielding about 10-fold less than wild-type cells. In contrast, the heat-stable A-factor activity in *asgE* cells is down only twofold compared with wild type. Thus, it appears that the primary defect of *asgE* cells is a lack of heat-labile A-factor. Previous studies have shown that heat-labile A-factor consists of at least two extracellular proteases (Plamann *et al.*, 1992). However, the fact that the proteases in pronase failed to rescue the sporulation deficiency of *asgE* cells suggests that the defects observed for the *asgE* mutant are not simply the result of lack of extracellular protease activity. For example, *asgE* cells may be defective for release of the A-signal proteases and their substrates. In any case, future work will be needed to help to determine the precise role of AsgE in the A-signal production pathway.

## Experimental procedures

### *Bacterial strains and plasmids*

A complete list of strains and plasmids used in this study is shown in Table 1.

### *Plasmid transfer to M. xanthus*

Plasmids containing DNA fragments from the *asgE* locus were electroporated into cells of DK101 or DK1622 using the technique of Plamann *et al.* (1994). After electroporation, cells were placed into flasks containing 1.5 ml of CTT broth and incubated at 32°C for 8 h with vigorous agitation. Aliquots (500  $\mu$ l) of these cultures were added to 5 ml of CTT soft agar and poured onto CTT plates containing kanamycin. Chromosomal DNA was isolated from Kan<sup>r</sup> colonies (Sambrook *et al.*, 1989) and used for Southern blot analysis (Sambrook *et al.*, 1989) to identify transformants that contain a single copy of the appropriate plasmid integrated by homologous recombination into the *asgE* locus. Kan<sup>r</sup> transformants carrying a single plasmid insertion were scored for development and sporulation efficiency as needed. To confirm that the developmental defects were caused by the plasmid insertions themselves, the insertions were transduced into parental strain DK101 or DK1622 using phage Mx8, and Kan<sup>r</sup> transductants were shown to have the same phenotype as the parental donor strain.

### *Media for growth and development*

*M. xanthus* strains were grown at 32°C in CTT broth containing 1% casitone (Difco Laboratories), 10.0 mM Tris

hydrochloride (pH 8.0), 1 mM KH<sub>2</sub>PO<sub>4</sub> and 8 mM MgSO<sub>4</sub>, or on plates containing CTT broth and 1.5% Difco bacto agar. CTT broth and CTT plates were supplemented with 40 µg ml<sup>-1</sup> kanamycin sulphate (Sigma) or 12.5 µg ml<sup>-1</sup> oxytetracycline (Sigma) as needed. CTT soft agar is CTT broth containing 0.7% Difco bacto agar. *Escherichia coli* strains DH5α and JM101 were grown at 37°C in LB containing 1% tryptone (Difco), 0.5% yeast extract (Difco) and 0.5% NaCl, or on plates containing LB and 1.5% Difco bacto agar. LB and LB plates were supplemented with 50 µg ml<sup>-1</sup> ampicillin (Sigma) or 40 µg ml<sup>-1</sup> kanamycin sulphate (Sigma) as needed. Fruiting body development was carried out at 32°C on TPM plates containing TPM buffer (10.0 mM Tris hydrochloride, pH 8.0, 1 mM KH<sub>2</sub>PO<sub>4</sub> and 8 mM MgSO<sub>4</sub>) and 1.5% Difco bacto agar. A-factor assays and pronase rescue experiments were performed in MC7 buffer (10 mM MOPS, 1 mM CaCl<sub>2</sub>, pH 7.0).

### *M. xanthus* development

*M. xanthus* strains were inoculated into a flask containing CTT, broth and the culture was placed at 32°C with vigorous swirling. After the culture reached a density of 5 × 10<sup>8</sup> cells ml<sup>-1</sup>, the cells were pelleted, the supernatant was removed, and the cells were resuspended in TPM buffer to a density of 5 × 10<sup>9</sup> cells ml<sup>-1</sup>. Aliquots (20 µl) of this cell suspension were spotted onto TPM agar plates and incubated at 32°C. The progress of fruiting body development was monitored visually using phase-contrast microscopy (Nikon Eclipse E800 microscope). Photographs were taken using an Optronics Engineering DEI-750T video camera and a Panasonic TQ3031F optical disc recorder.

To determine the sporulation efficiency of *M. xanthus* strains, developing cells were harvested from TPM agar plates after 5 days as described previously (Kroos *et al.*, 1986). The cells were resuspended in 400 µl of TPM buffer, and the cell suspension was sonicated and incubated for 2 h at 50°C. The number of heat- and sonication-resistant spores that germinated into colonies was determined as described by Thöny-Meyer and Kaiser (1993).

For extracellular complementation of development, vegetatively growing cells carrying mutations in the *asgE* locus were concentrated to a density of 5 × 10<sup>9</sup> cells ml<sup>-1</sup> as described above, mixed at a 1:1 ratio with wild-type cells, and 20 µl aliquots of the cell mixtures were spotted onto TPM agar plates. For complementation experiments with *asg*, *csg* or *esg* strains, cells were mixed at 1:1 ratio. The sporulation efficiency for each strain in the mixed culture fruiting body was determined as described above. Because the Ω5003 insertion in *asgE* creates a transcriptional fusion with *lacZ*, colonies derived from MS2021 were distinguished from the colonies derived from other strains because they become dark blue rapidly after overlaying with CTT soft agar containing Xgal. Colonies derived from the MS2020 mutant were distinguished from wild-type colonies based on their resistance to kanamycin.

### (p)ppGpp assays

Guanosine nucleotides were isolated and measured as

described previously (Manoil and Kaiser, 1980; Singer and Kaiser, 1995).

### A-factor assays

A-factor assays were performed using *M. xanthus* strain DK4323. A-factor was provided by cell-free MC7 buffer conditioned by strain DK101, strain DK476 or strain MS2021 as described previously by Plamann *et al.* (1992).

### Phenotypic rescue of mutants

Pronase rescue experiments were carried out in submerged culture at a density of 5 × 10<sup>9</sup> cells ml<sup>-1</sup> as described by Kuspa *et al.* (1992a). The cell suspension was supplemented with 40 µg ml<sup>-1</sup> pronase as needed, and the sporulation efficiencies for mutant and wild-type cells after 5 days of development were determined as described above. To examine the effect of cell density on the sporulation efficiency of *asgE* mutant, MS2021 cells were grown in CTT broth to a density of 5 × 10<sup>8</sup> cells ml<sup>-1</sup>, the cells were pelleted, the supernatant was removed, and the cells were resuspended in the appropriate amount of TPM buffer to give the desired cell density. Cells were spotted onto TPM agar plates, and the sporulation efficiencies were determined as described above for developmental assays.

### DNA sequence analysis

DNA was sequenced by the dideoxynucleotide chain termination method (Sanger *et al.*, 1977) using the Sequi-Therm Cycle Sequencing Kit (Epicentre Technologies) and custom-designed oligonucleotide primers synthesized by Operon Technologies. The nucleotide sequence of both strands of a 6.0 kb region in the *asgE* locus (formerly called the *sdeAB* locus) was determined in this manner. Approximately 500 bp at the 3' end of this 6.0 kb of sequence overlaps with the DNA sequence described by Harris and Singer (1998). The DNA sequence was analysed using ABI PRISM software and assembled using Deneba SEQUENCHER software. Potential membrane-spanning regions in protein coding sequences were identified using the TMFPRED program.

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