A gene encoding a potential adenosine 5′-phosphosulphate kinase is necessary for timely development of Myxococcus xanthus

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INTRODUCTION

The model organism Myxococcus xanthus serves as a prototype for research in prokaryotic development. Under conditions of nutrient starvation on a solid surface, a developmental programme is initiated that results in cell aggregation and sporulation. The sporulation programme controls the differentiation of rod-shaped cells into dormant spherical spores that are surrounded by thick spore coats and are stress resistant (Dworkin, 1996; Konovalova et al., 2010). The cell wall-like spore coat consists primarily of carbohydrates (Holkenbrink et al., 2014; Kottel et al., 1975) and a number of proteins, including proteins C, S and U, forming a cuticle around the polysaccharide layer (Dahl et al., 2007; Dworkin 1996; Otani et al., 1998; Tengra et al., 2006). For spore coat precursor proteins bearing a classical N-terminal signal peptide, it is thought that they are translocated across the inner membrane by the general secretory (Sec) machinery and then translocated across the outer membrane by either the main terminal branch or one of several protein-specific export systems (Saier, 2006). Among protein-specific secretion systems that function exclusively in export across the outer membrane, the chaperone–usher (CU) pathway is a conserved system for assembly and secretion of a large family of adhesive protein polymers termed pili or fimbriae at the cell surfaces of Gram-negative bacteria (Geibel & Waksman, 2013; Waksman & Hultgren, 2009). Previously we demonstrated that McuA (MXAN3885), a structural component of the M. xanthus CU system (MXAN3885–MXAN3882), is assembled on spore surfaces and hence considered to be a spore coat protein (Leng et al., 2011). With the help of the periplasmic chaperone McuB, the structural subunit McuA, together with another subunit McuD, is polymerized and exported through the putative outer membrane usher McuC by a donor strand exchange mechanism (Wu et al., 2014; Zhu et al., 2013). Thus, besides involvement in pilus biogenesis, CU secretion systems also play a role in spore coat formation.

Aggregation and sporulation, the two morphogenetic processes underlying fruiting body formation, are tightly

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Abbreviations: APS, adenosine 5′-phosphosulphate; CU, chaperone–usher; ONP, o-nitrophenol; PAPS, 3′-phosphoadenosine 5′-phosphosulphate; PDB, Protein Data Bank.
coordinated via C-signal, a cell surface-associated protein. Aggregation is induced after 6 h whereas sporulation is not initiated until the aggregation process is complete at 24 h. Fruiting body morphogenesis also involves temporally and spatially coordinated changes in gene expression. Upon entry to the developmental stage, many genes are sequentially turned on at specific time points in cells that are densely packed inside the fruiting bodies (Søgaard-Andersen et al., 2003). Generally, genes preferentially expressed in sporulating cells are turned on after 6 h and depend on aggregation of cells into mounds. According to the current model for C-signal-dependent coordination of aggregation and sporulation, only cells that have accumulated inside the mounds reach the high level of C-signalling required for later gene expression and spore differentiation (Søgaard-Andersen et al., 2003). Consistent with this, expression of the mculABC operon, a main part of the M. xanthus mcul gene cluster, depends on C-signalling and cell aggregation, and mculABC expression is reduced in any strain in which aggregation is delayed (Cao, P. et al., 2015a).

Identification of players important for development has been one of the central themes for the biology of myxobacteria. Although our understanding of the developmental programme is far from complete, it is clear that building a multicellular fruiting body with a defined size and shape requires a regulatory network that is highly responsive to environmental, intercellular and intracellular signals (Kaiser et al., 2010). Through extensive genetic and biochemical studies, a number of factors thought to be involved in cellular aggregation and sporulation have been identified, including sigma factors, two-component proteins, σ4-dependent enhancer-binding proteins, serine/threonine kinases, proteases, and even non-coding RNA and secondary metabolites (Kaiser et al., 2010; Meier et al., 2006; Yu et al., 2010). In this work, we initially intended to search for regulatory genes potentially involved in controlling mcul expression; however, using transposon mutagenesis we identified MXAN3487 (MXAN_RS16905), a gene encoding a putative adenosine 5′-phosphosulphate (APS) kinase (i.e. adenylylsulphate kinase), as a regulator for the timing of cell aggregation. Loss of MXAN3487 function caused defects in fruiting, leading to a secondary effect on the timing of mculABC expression.

**METHODS**

**Cell growth, development, sporulation assay and measurement of β-galactosidase activity.** *Escherichia coli* strains were grown in LB medium in the presence of relevant antibiotics. *M. xanthus* strains were grown in CTT liquid medium (1 % casitone, 8 mM MgSO₄, 10 mM Tris/HCl, 1 mM potassium phosphate, pH 7.6) and CTT agar (1.5 %) plates. When required, kanamycin or oxytetracycline was used at concentrations of 40 and 12.5 μg/ml, respectively. *M. xanthus* development was induced by spotting 20 μl aliquots (4 × 10⁷ cells ml⁻¹) on TPM plates (10 mM Tris/HCl, 1 mM KH₂PO₄, 8 mM MgSO₄, pH 7.6, 1.5 % Difco Bacto-Agar). TPM containing 8 mM MgCl₂ instead of MgSO₄ was used to simulate sulphate starvation. The efficiency of sporulation was measured as described previously (Gorski & Kaiser, 1998). For quantitative lacZ reporter assays, two PCR-verified independent transformants for each strain were chosen randomly and specific β-galactosidase activities were measured as described previously (Kroos et al., 1986). Protein concentrations were measured by a BCA Protein Assay kit (Pierce).

**M. xanthus strains.** *M. xanthus* strains used in this study are listed in Table 1. DK1622 (Kaiser, 1979) was used as the parent WT strain for all *M. xanthus* strains constructed in this study. All strains constructed were confirmed by PCR.

**Transposon mutagenesis.** The plasmid p15A-MycocMar-oriT-R6K-kky-neo, which carries the MycoMar transposable element of the mariner family (Rubin et al., 1999), was used to transform DK1622/pMP-mculABC, an mculABC-lacZ transcriptional fusion strain. Transformation, screening and determination of the transposon insertion location were carried out as described previously (Cao, S. et al., 2015).

**Construction of MXAN3487–lacZ fusion.** MXAN3487–lacZ transcriptional fusion was constructed using the vector pMP220 (Spaenkötter et al., 1997). A 0.86 kb DNA fragment containing part of the MXAN3487 5′ coding sequence and its putative promoter region was generated by PCR using DK1622 genomic DNA as the template. The PCR primer pair was MXAN3487-5 and MXAN3487-3, spanning nucleotides between 816 bp upstream and 44 bp downstream of the MXAN3487 start codon. The PCR product obtained was digested with XbaI/PstI, cloned into the corresponding sites in the pBluescript vector and verified by DNA sequencing. Then, the plasmid-derived Xba/Pst fragment, together with a 2.9 kb EcoRI/XbaI myxophase Mx₈ attP fragment recovered from a pUC18 derivative carrying Mx₈ attP, were ligated with pMP220 that had been digested with EcoRI/PstI, yielding a transcriptional lacZ fusion vector pMP-MXAN3487. The plasmid was then introduced into *M. xanthus* through electroporation (Kashefi & Hartzell, 1995). Transformants were selected by plating the cells onto CTT plates containing oxytetracycline. The plasmid used in this study could not independently replicate out of the *M. xanthus* chromosome. Thus, all antibiotic-resistant transformants resulted from integration of the plasmid into the chromosome either by homologous recombination or by site-specific recombination between attP on the plasmid and the attB site on the chromosome. PCR was performed to screen antibiotic-resistant colonies for proper integration of each plasmid at attB using the following primer pair: forward, 5′-GAAGGCGCGGAACTTTGGATCCGGG-3′; reverse, 5′-CCATGACGGAGAGGCTGCGAGGT-3′.

**Immunoblot analysis.** To analyse accumulation of McuA, *M. xanthus* strains were grown in CTT to OD₆₀₀ 0.8 (4 × 10⁸ cells ml⁻¹). Cells were sedimented and resuspended in 1/10 vol. TPM buffer. Aliquots (20 μl) of concentrated cells were spotted on TPM or modified TPM agar plates. Developmental cells were harvested at the indicated times and protein samples were prepared as described previously (Leng et al., 2011; Zhu et al., 2013). For each sample a 5 μl aliquot representative of an equal number of cells (4 × 10⁷ cells) at the beginning of development was applied per lane. Blots were probed with rabbit anti-McuA serum, followed by anti-rabbit IgG conjugated to alkaline phosphatase with the chromogenic substrates nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

**Construction of MXAN3487 in-frame deletion.** Plasmid pBJ113-ΔMXAN3487 is a pBJ113 derivative (Julien et al., 2000) generated to create a MXAN3487 deletion mutation. To construct pBJ113-ΔMXAN3487, a 1 kb upstream and a 1.1 kb downstream fragment flanking MXAN3487 were PCR-amplified using the primer pairs H1/HindIII and H2/HsamHI/H2/EcoRI, respectively (Table 2). The two

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amplified products were digested, ligated and cloned into pBJ113 to obtain the plasmid pBJ113-ΔMXAN3487. After verification by DNA sequencing, the plasmid was introduced into M. xanthus DK1622 by electroporation and transformants were selected on CTT plates containing 40 μg ml−1. Individual kanamycin-resistant transformants were then grown in CTT broth in the absence of kanamycin and plated onto CTT plates supplemented with 1 % galactose for negative selection. PCR products were used to screen galactose-resistant and kanamycin-sensitive colonies for proper excision of the WT copy as described previously (Shi et al., 2008). The resulting strain ΔMXAN3487 contained a 552 bp in-frame deletion corresponding to codons 2–185 in the 185 aa MXAN3487 protein.

Expression of WT and mutant MXAN3487 genes in the strain ΔMXAN3487. To construct the plasmid expressing WT MXAN3487 protein, a 1.4 kb fragment encompassing the full-length MXAN3487 ORF and its putative promoter sequence was amplified using the primer pair MXAN3487FL-S1–3 and the DK1622 chromosome as a template. Generation of a K19R point mutation was carried out by the method of two-step PCR as described previously (Shi et al., 2008). All final PCR products were gel purified, digested with XbaI/PstI and ligated to pBluescript cut with the same enzymes, followed by transformation of E. coli JM83. The fragments representing WT and mutant MXAN3487 genes were confirmed by DNA sequencing. Subsequently, the 1.4 kb XbaI/PstI fragment of WT MXAN3487 or its mutant version, together with a 2.9 kb EcoRI/XbaI myxophage Mx8 attP fragment, was cloned into the EcoRI/PstI sites of pMP220 to create pMP-X, where X denotes either a full-length WT (MXAN3487FL) or the K19R mutant gene. For expression of WT and mutant MXAN3487 proteins, the resulting plasmids were separately introduced into ΔMXAN3487 by electroporation. Proper integration of each plasmid at attB was verified by PCR.

Expression of the E. coli cysC gene in the strain ΔMXAN3487. A 0.82 kb fragment containing the MXAN3487 promoter region and start codon was amplified using the primers MXAN3487H1–5 and MXAN3487H1 BamHI, with the M. xanthus chromosome as a template. The PCR product thus obtained was digested with XbaI/BamHI. Full-length E. coli cysC ORF (0.6 kb) was amplified using the primer pair cysC-5/-3, and E. coli chromosome as a template. The resulting PCR product was digested with BamHI/PstI. These two fragments were ligated with pBluescript that had been cut with XbaI/PstI. Clones containing the correct insert were verified by DNA sequencing. Subsequently, the 1.4 kb XbaI/PstI insert together with a 2.9 kb EcoRI/XbaI myxophage Mx8 attP fragment was cloned into the EcoRI/PstI sites of pMP220. The resulting plasmid was introduced into ΔMXAN3487 by electroporation. Proper integration of this plasmid at attB was verified by PCR.

Homology modelling and multiple sequence alignment. The Phyre2 web portal (Kelley et al., 2015) was used to construct a homology model for MXAN3487. Multiple sequence alignment was carried out using the CLUSTAL Omega web server (McWilliam et al., 2013) (http://www.ebi.ac.uk/Tools/msa/clustalo/).

RESULTS

Identification of MXAN3487 as a gene that impacts mcuABC expression

Genome-wide transposon mutagenesis was carried out in the mcuABC–lacZ fusion strain on TPM plates containing X-Gal substrate, as described previously (Cao, S. et al., 2015). Clones not appearing blue were identified and the
position of the transposon was mapped. In one clone the transposon was located in the region encompassing MXAN3487, and diminished MucA accumulation in this transposon mutant was confirmed by Western blot analysis (data not shown). To confirm the possibility that MXAN3487 might impact mcuABC expression, we constructed the strain ΔMXAN3487, a MXAN3487 in-frame deletion mutant. In contrast to the WT where MucA accumulation was observed at all developmental time points examined, MucA was not detectable until 72 h when MXAN3487 was deleted. The discrepancy between mcuABC transcription and MucA accumulation at 48 h in the ΔMXAN3487 strain presumably reflects post-transcriptional regulation of mcuABC at a later developmental stage. To ascertain that lack of MucA in the ΔMXAN3487 mutant was indeed caused by the loss of MXAN3487 function, a plasmid (pMP-MXAN3487) carrying a MXAN3487–lacZ transcriptional fusion was constructed. This fusion construct contained a full-length ORF of WT MXAN3487 together with its upstream regulatory sequence and integrated at the attB of the ΔMXAN3487 chromosome. As shown in Fig. 1(c), with respect to the level of MucA, strain ΔMXAN3487 was successfully complemented by ectopic expression of MXAN3487. Thus, transposon mutagenesis identified MXAN3487 as a gene necessary for appropriate mcu expression during M. xanthus development.

### Delayed expression of mcuABC in ΔMXAN3487 may be secondary to a cell aggregation defect

The M. xanthus mcu gene cluster functions in spore coat formation (Leng et al., 2011). According to the current model of fruiting body morphogenesis (Søgaard-Andersen et al., 2003), genes that are specifically expressed in sporing cells depend on aggregation of cells into mounds. In addition, we have demonstrated the absence of MucA in glycerol-induced spores, where sporulation is uncoupled from cell aggregation (Cao, P. et al., 2015). Therefore, a possible explanation for delayed expression of mcuABC in ΔMXAN3487 is that it might be due to defective cell aggregation. In view of this, the strain ΔMXAN3487 was assayed for developmental phenotypes by spotting concentrated cell suspensions on TPM starvation agar. For the WT strain DK1622, translucent mounds were evident at 6–12 h after the onset of development (Fig. 2). In contrast to the WT, ΔMXAN3487 remained at the loose and elongated mounds stage of translucent mounds as late as 48 h (Fig. 2). At 72 h, the loose and elongated mounds did not completely condense or darken into mature fruiting bodies, although condensed and darkened fruiting bodies were visible.
after 7 days of starvation. The ΔMXAN3487 mutant was also defective for spore viability, with \( < 1 \times 10^{-4} \% \) viable spores produced compared with the WT (Table 3). Note that the aggregation and sporulation defects could be corrected by introducing a MXAN3487-expressing plasmid into the strain ΔMXAN3487 (Fig. 2, Table 3), providing evidence that the developmental defects of ΔMXAN3487 cells were caused by loss of MXAN3487 function. Notably, the timing of mcuABC expression in the ΔMXAN3487 strain agreed well with that of cell aggregation (Figs 1 and 2). The observed correlation between mcuABC expression and cell aggregation in ΔMXAN3487 supported the hypothesis that MXAN3487 was involved in regulating the timing of entry into development, which is required for timely expression of mcuABC.

M. xanthus is a gliding bacterium that possesses two motility systems, the adventurous (A)- and social (S)-motility systems (Cao, P. et al., 2015; Islam & Mignot, 2015), both of which are necessary for aggregation of cells into mounds. To assess A- and S-motility in the ΔMXAN3487 strain, motility was tested on soft (0.5 %) agar, which favours S-motility, and hard (1.5 %) agar, which favours A-motility (Shi & Zusman, 1993). Compared with DK1622, the ΔMXAN3487 strain did not display compromised S- or A-motility (data not shown).

Expression of MXAN3487 is induced during development and depends on FruA

As MXAN3487 is important for timely fruiting body formation, we were interested in determining whether expression of MXAN3487 was developmentally regulated. For this purpose, a plasmid (pMP-MXAN3487) carrying a MXAN3487–lacZ transcriptional fusion was introduced into the WT strain DK1622. As shown in Fig. 3, β-galactosidase activity was not detectable within 12 h after starvation-initiated development, but started to appear at 15 h at a very low level, markedly increased at 18 and 24 h, and remained high over the next 48 h. Therefore,
like mcuABC, expression of MXAN3487 was specifically induced during development.

Most M. xanthus genes turned on after 6 h are induced by C-signalling (Søgaard-Andersen et al., 2003). To test if MXAN3487 expression followed this rule, we examined whether FruA, a key response regulator protein in the C-signalling pathway, was required for MXAN3487 transcription. For this purpose, a plasmid carrying a MXAN3487–lacZ transcriptional fusion was introduced

Fig. 2. Developmental phenotypes of the ΔMXAN3487 strain complemented with WT MXAN3487, a MXAN3487 mutant allele (MXAN3487-K19R) or the E. coli cysC gene encoding an APS kinase. Cells were starved on TPM agar for the indicated periods of time and examined under a Nikon SMZ1500 stereomicroscope. DK1622, WT M. xanthus.
MXAN3487 encodes a putative APS kinase

A BLAST (Altschul et al., 1990) search with the MXAN3487 sequence revealed high sequence similarity to proteins containing an APS kinase domain. Using Phyre2 (Kelley et al., 2015), six templates [Protein Data Bank (PDB) IDs: 1xjq (chain A), 2gks (chain B), 1m8p (chain B), 1m7g (chains A and B), and 1xnj (chain B)] with 39–50 % sequence identity to MXAN3487 were selected to maximize confidence, percentage identity and alignment coverage, resulting in a homology model of MXAN3487 with >90 % confidence. All of the templates were APS kinase domains except for PDB ID: 1m8pB, which is the APS kinase-like allosteric domain of Penicillium chrysogenum ATP sulphonylase (MacRae et al., 2002). A CLUSTAL Omega (McWilliam et al., 2013) multiple sequence alignment of MXAN3487, the Phyre2 templates and E. coli APS kinase CysC are shown in Fig. 4(a). As expected for an APS kinase, the MXAN3487 sequence harboured a Walker A (P-loop) ATP-binding motif (Saraste et al., 1990) near the N terminus and a sulphonucleotide-binding motif (consensus sequence AXGXIXXFTG) (Lansdon et al., 2002) towards the C-terminal region. The homology model showed that MXAN3487 had a typical APS kinase active site (Fig. 4b, c), including the conserved Arg (R121) in the mobile lid region of APS kinases that was absent from the non-ATP-binding allosteric domain of P. chrysogenum ATP sulphonylase, suggesting that MXAN3487 was a bona fide APS kinase.

To ascertain whether the MXAN3487 protein functions as an active kinase in vivo, we constructed an MXAN3487 mutant allele in which the P-loop Lys was substituted by Arg (K19R). K19R is a kinase-dead point mutation if the MXAN3487 protein functions as a kinase, because the Lys residue in the P-loop is required for productive ATP-binding and kinase activity (Deyrup et al., 1998). A plasmid carrying the mutant allele (pMP-MXAN3487-K19R) was introduced into ΔMXAN3487 and integrated at the chromosomal attB. As shown in Fig. 2, in contrast to the WT, the mutant allele was unable to complement the aggregation defect of the strain ΔMXAN3487 up until 36 h of development, and at 48 and 72 h, it only partially ameliorated the aggregation defect of ΔMXAN3487 because the complementary strain formed loose and elongated assemblages of cells that did not condense or darken into mature fruiting bodies. Moreover, the K19R allele was unable to rescue the sporulation defect of ΔMXAN3487 (Table 3). Meanwhile, we carried out immunoblot analyses of total proteins isolated from developing ΔMXAN3487 cells containing either MXAN3487 WT or its K19R mutant version. With respect to the level of McuA, the K19R allele failed to rescue McuA accumulation at 18 and 24 h (Fig. 5a). The MXAN3487 homology model suggested that it was unlikely the K19R mutation would result in structural complications leading to an unstable protein, and we concluded that the kinase activity of MXAN3487 protein is essential for its function.

We further explored whether MXAN3487 had APS kinase activity in vivo. The function of MXAN3487 was tested by complementation of the strain ΔMXAN3487 with a plasmid expressing a well-known APS kinase-encoding gene, the cysC gene of E. coli (Leyh et al., 1992). We observed that E. coli cysC partially complemented the aggregation defect and McuA accumulation deficiency in ΔMXAN3487 at 48 h post-starvation (Figs 2 and 5b). At 168 h, the fruiting body morphology of ΔMXAN3487/cysC more closely resembled the WT than did the morphology of ΔMXAN3487 or ΔMXAN3487/MXAN3487-K19R (Fig. 2). Moreover, the ability of ΔMXAN3487/cysC to sporulate was ~150-fold higher than the sporulation efficiency of ΔMXAN3487 (Table 3). These results indicate

Table 3. Spore viability assay

<table>
<thead>
<tr>
<th>Strain</th>
<th>Percentage of WT sporulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>DK1622</td>
<td>100</td>
</tr>
<tr>
<td>ΔMXAN3487</td>
<td>&lt;10^-4</td>
</tr>
<tr>
<td>ΔMXAN3487/cysC</td>
<td>0.015</td>
</tr>
<tr>
<td>ΔMXAN3487/MXAN3487-K19R</td>
<td>&lt;10^-4</td>
</tr>
<tr>
<td>ΔMXAN3487/MXAN3487</td>
<td>98</td>
</tr>
</tbody>
</table>

Spore counts <10 per experiment were undetectable by our method. Sporulation frequencies are presented relative to the level of sporulation in DK1622 as the means of triplicate samples. For each sample, a total of 4 x 10^8 cells were starved on TPM agar for 72 h.

Fig. 3. Expression of MXAN3487 is developmentally regulated and depends on FruA. Specific activity of β-galactosidase expressed from a MXAN3487-lacZ transcription fusion during development was measured in WT (DK1622) and fruA mutant backgrounds. At various time points the cells were harvested and samples were analysed as described in the legend to Fig. 1(b). Data represent mean±SD derived from triplicate samples of a representative transformant. Two independent transformants of each strain were assayed, with similar results.
Fig. 4. (a) Multiple sequence alignment of MXAN3487 with APS kinases and one ATP sulphurylase non-ATP-binding allosteric domain. Ec APSK, E. coli APS kinase (GenBank accession number ALL89231.1); Hu APSK, APS kinase domain of human PAPS synthetase 1 (PDB ID: 1XJQ); Aa APSK, APS kinase domain of Aquifex aeolicus bifunctional ATP sulphurylase/adenosine 5’-phosphosulphate kinase. (b) Structural model of MXAN3487 showing Walker A and Sulphonucleotide-binding motifs. (c) Sulphonucleotide-binding motif of MXAN3487.
sulphurylase-APS kinase (PDB ID: 2GKS); Pc APSK, _P. chrysogenum_ APS kinase (PDB ID: 1M7G); Pc ATPS, allosteric domain of _P. chrysogenum_ ATP sulphurylase (PDB ID: 1M8P). Secondary structure elements in the homology model of _M. xanthus_ APS kinase are shown as cylinders (helices) and arrows (β-strands) below the sequences. Identical residues in the APS kinases are shown in bold. ATP-binding residues are shown in magenta and APS-binding residues in green. The mobile lid region is boxed. An arrow points to the mobile lid Arg residue that is conserved in APS kinases but missing from the non-ATP-binding allosteric domain of _P. chrysogenum_ ATP sulphurylase (Lansdon et al., 2002). (b) Surface (semi-transparent) and cartoon representations of the MXAN3487 homology model with ADP and APS (sticks) from the X-ray structure of _P. chrysogenum_ APS kinase (PDB ID: 1M7G) superimposed. (c) Close-up of the active site in the homology model of MXAN3487, with ADP and APS from the X-ray structure of _P. chrysogenum_ APS kinase (PDB ID: 1M7G) superimposed. The view is essentially the same as in (b).

potential APS kinase activity of the MXAN3487 protein. A possible explanation for weak complementation of ΔMXAN3487 could be weak expression of _E. coli_ cysC in _M. xanthus_ due to codon preference (Romeo et al., 1986).

**MXAN3487 is not regulated by sulphur starvation**

APS kinase catalyses the second reaction in the two-step conversion of inorganic sulphate to 3'-phosphoadenosine 5'-phosphosulphate (PAPS) (MacRae et al., 2000; Williams et al., 2002). In many bacteria, PAPS is the substrate for a reductive assimilation pathway leading to the biosynthesis of cysteine and, eventually, to other reduced sulphur-containing metabolites. PAPS is an activated sulphate form that is also used by the cell to create a variety of sulphated metabolites. Thus, it is reasonable that APS genes are subject to regulation by sulphur availability. For instance, the expression of the _Mycobacterium tuberculosis_ APS kinase-encoding gene cysC was reported to be induced by sulphate deficiency and repressed by the presence of cysteine (Pinto et al., 2004). To study whether MXAN3487 expression was regulated by sulphur availability, we tested the expression pattern of a chromosomally located MXAN3487–lacZ transcriptional fusion under sulphate starvation conditions. With respect to both timing and level of expression (Fig. 6a), the specific activity of β-galactosidase in cells that developed in sulphur-free medium was indistinguishable from that in sulphur-containing medium. In accordance with this, accumulation of McuA in the WT strain in the absence of sulphate was comparable to that in the presence of sulphate (Fig. 6b). Therefore, in contrast to _M. tuberculosis_ cysC, MXAN3487 was not regulated by sulphur availability during _M. xanthus_ development.

**DISCUSSION**

The _M. xanthus_ mcuABCD gene cluster is involved in spore coat formation (Leng et al., 2011). This cluster is the only locus coding for a CU system in this bacterium and its function is apparently distinct from most fimbrial CU systems in other bacteria (Waksman & Hultgren, 2009). In an effort to better understand developmentally regulated expression of the Mcu system, we undertook a transposon mutagenesis approach to identify regulators of the mcuABC operon. Previously, we demonstrated that MXAN2872, a gene encoding a putative FAD-binding monooxygenase, plays a positive role in controlling _M. xanthus_ development and mcuABC expression (Cao, S. et al., 2015). Based on the presence of a typical APS kinase active site in the homology model of MXAN3487, its _in vivo_ APS kinase activity and the importance of the ATP-binding sequence for activity, it seems likely that MXAN3487 functions as an APS kinase. The agreement between the timing of McuA accumulation and that of developmental progression of two MXAN3487 mutants (ΔMXAN3487 and ΔMXAN3487/MXAN3487-K19R) reinforces the idea that mcuABC expression is morphogenesis dependent. Thus, similar to MXAN2872, MXAN3487 modulates the onset of cell agglutination, which in turn triggers mcuABC expression.
It is worth mentioning that after 7 days of starvation \( \Delta MXAN3487 \) and \( \Delta MXAN3487/\text{MXAN3487-K19R} \) were still able to produce fruiting bodies, albeit delayed and irregularly shaped.

How the MXAN3487-encoded APS kinase modulates \( M. \text{xanthus} \) development and consequently affects \( mcuABC \) expression is currently unknown. Several lines of evidence suggest that the MXAN3487-encoded APS kinase is unlikely to use APS to produce PAPS for the biosynthesis of cysteine, a major end-product of sulphur assimilation. (1) Expression of \( mcuABC \) and the MXAN3487 itself is not affected by sulphur limitation. (2) Unlike many APS kinase genes in other bacteria (Leyh et al., 1992; Pinto et al., 2004; Shen et al., 2002), MXAN3487 does not lie close to the ATP sulphurylase-coding genes MXAN2336 (\( \text{cysN} \)) and MXAN2337 (\( \text{cysD} \)) whose products form a heterodimer responsible for activating sulphate, the first step in the sulphate assimilation pathway. (3) MXAN2336 and MXAN2337 are the only neighbouring genes encoding the two subunits of \( M. \text{xanthus} \) ATP sulphurylase, yet MXAN2336 is not expressed during development (data not shown), implying that under starvation conditions the cysteine biosynthesis pathway may be inactive. The cysteine needed for the synthesis of proteins and other reduced sulphur-containing compounds may, for instance, come from the products generated by developmental autolysis of vegetative cells. Alternatively, \( M. \text{xanthus} \) may use another putative APS kinase encoded, for instance, by the ‘orphan’ APS kinase genes MXAN3487 and MXAN4064 in \( M. \text{xanthus} \), although it remains unknown.

In addition to catalysing the phosphorylation of APS to form PAPS, APS kinase also phosphorylates the 3'-hydroxyl group of the ribose ring of molecules that are structurally related to APS, including dephospho-CoA (Satishchandran et al., 1993). Phosphorylation of dephospho-CoA is the last step in the biosynthetic pathway of CoA (Begley et al., 2001), a principal carrier for acetyl and acyl groups and important for the biosynthesis of fatty acids, polypeptide antibiotics and polyketides. Notably, some metabolites (e.g. isoprenoids and Dkxanthene) whose production depends on CoA are essential for \( M. \text{xanthus} \) development (Lorenzen et al., 2009; Meiser et al., 2006, 2008). In view of this, we propose that under starvation conditions the MXAN3487-encoded APS kinase may participate in CoA biosynthesis and consequently affect CoA-dependent production of biomolecules that might serve as structural components and signalling compounds necessary for \( M. \text{xanthus} \) development. This view was supported by the appearance of two relevant genes immediately upstream of MXAN3487: MXAN3485 and MXAN3486, which encode proteins with homology to 4'phosphopantetheinyl transferase and ppa00975 thioesterase domain protein, respectively. Both 4'-phosphopantetheinyl transferase and thioesterase catalyse CoA-dependent biochemical reactions. Another line of evidence comes from undetectable changes in MXAN3487 expression in response to sulphur availability, consistent with the observation in \textit{Pseudomonas aeruginosa} that production of an APS kinase possibly involved in CoA synthesis is not repressed by the presence of cysteine (Satishchandran et al., 1993).

Finally, it is noteworthy that MXAN3487 is not expressed in vegetative cells (Fig. 3). Thus, the present data do not support the involvement of MXAN3487 in sulphur assimilation in the complex life cycle of \( M. \text{xanthus} \), although it may function as an APS kinase and is required for proper development of this bacterium.

Fig. 6. (a) Expression of MXAN3487-lacZ fusion in response to sulphur stimulus. \( M. \text{xanthus} \) DK1622 cells carrying the MXAN3487-lacZ operon fusion were plated on starvation agar in the presence (+SO\(_4^{2-}\)) or absence (−SO\(_4^{2-}\)) of sulphate. At various time points the cells were harvested and samples were analysed as described in the legend to Fig. 1(b). Data represent mean±SD derived from triplicate samples of a representative transformant. Two independent transformants of each strain were assayed with similar results. (b) Accumulation of McuA in response to sulphur stimulus. DK1622 cells were plated on starvation agar in the presence (+SO\(_4^{2-}\)) or absence (−SO\(_4^{2-}\)) of sulphate. At various time points the cells were harvested and then analysed by Western blotting as described in the legend to Fig. 1.
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