**HthA, a putative DNA-binding protein, and HthB are important for fruiting body morphogenesis in *Myxococcus xanthus***

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In response to starvation, *Myxococcus xanthus* initiates a developmental programme that results in the formation of spore-filled multicellular fruiting bodies. Fruiting body formation depends on the temporal and spatial coordination of aggregation and sporulation and involves temporally and spatially coordinated changes in gene expression. This paper reports the identification of two genes, *hthA* and *hthB*, that are important for fruiting body formation. *hthA* and *hthB* are co-transcribed, and transcription of the two genes decreases strongly during development. Loss of *HthA* and *HthB* function results in delayed aggregation, a reduction in the level of sporulation, and abnormal developmental gene expression. Extracellular complementation experiments showed that the developmental defects caused by loss of *HthA* and *HthB* function are not due to the inability to synthesize an intercellular signal required for fruiting body formation. *HthA*, independent of *HthB*, is required for aggregation. *HthB*, alone or in combination with *HthA*, is required for sporulation. *HthA* is predicted to contain a C-terminal helix–turn–helix DNA-binding domain. Intriguingly, the N-terminal part of *HthA* does not exhibit significant amino acid similarity to proteins in the databases. The *HthB* protein lacks homologues in the databases. The results suggest that *HthA* is a novel DNA-binding protein, which regulates transcription of genes important for aggregation, and that *HthB*, alone or in combination with *HthA*, stimulates sporulation.

**INTRODUCTION**

Bacteria are frequently challenged by drastic changes in their extracellular environment. Successful competition and survival depend on adaptive responses to the altered conditions. To deal with starvation, *Myxococcus xanthus* cells have adopted a highly complex survival strategy, which ultimately results in the formation of spore-filled fruiting bodies. Fruiting body morphogenesis involves the temporal and spatial coordination of two morphogenetic processes: aggregation of cells into fruiting bodies, and sporulation. In the presence of nutrients, the rod-shaped motile *M. xanthus* cells grow and divide. *M. xanthus* cells move by gliding (Spormann, 1999) and if cells are present on a solid surface they form cooperatively feeding swarms. In response to starvation at a high density on a solid surface, cells initiate the developmental programme that culminates in the formation of the spore-filled fruiting bodies (Dworkin, 1996). The first morphological signs of fruiting body formation are evident at 6 h, with changes in cell behaviour and the formation of small aggregation foci (Jelsbak & Søgaard-Andersen, 2003). As more cells aggregate into these foci, they increase in size and become mound shaped. Eventually, a mound holds $10^5$ densely packed cells. Inside the mounds the rod-shaped cells differentiate to spores, resulting in mature fruiting bodies. Whereas mound formation is complete after 24 h, spore-maturation is complete after 72–120 h of starvation. Cells that remain outside the fruiting bodies differentiate into a cell type called peripheral rods, which is physiologically distinct from vegetative rods (Julien et al., 2000; O’Connor & Zusman, 1991a, b).

Fruiting body morphogenesis involves temporally coordinated changes in gene expression in which genes are turned on at specific time points during development (Inouye et al., 1979; Kroos et al., 1986). Moreover, for several genes that are turned on after 6 h, induction is tied to the spatial position of the cells, i.e. these genes are expressed in cells...
that aggregate and which eventually differentiate into spores, whereas they are not expressed in cells that differentiate to peripheral rods (Julien et al., 2000). In contrast, genes activated prior to 6 h are expressed in all cells, including peripheral rods (Julien et al., 2000). Three lines of evidence suggest that linked regulatory pathways coordinate morphogenesis and developmental gene expression (Søgaard-Andersen et al., 2003). Firstly, most developmental mutants display defects in morphogenesis as well as in developmental gene expression (Dworkin, 1996). Secondly, in several cases, developmentally regulated genes not only direct the expression of downstream developmental genes, but are also important for morphogenesis (e.g. Kroos et al., 1990). Finally, the cell-position-specific expression of genes turned on after 6 h indicates that cells are able to detect their position during fruiting body formation and tailor their gene expression profile accordingly.

During fruiting body formation, cells interact with each other using at least five intercellular signals (A to E) (Shimkets, 1999). Analyses of developmental gene expression in signalling mutants suggest that the signalling systems are arranged in a time-based hierarchy and that they lie on the same developmental pathway (Cheng & Kaiser, 1989; Downard & Toal, 1995; Gill & Cull, 1986; Kroos & Kaiser, 1987; Kuspa et al., 1986). Mutants deficient in any of the signalling systems are deficient in aggregation and sporulation, and display abnormal developmental gene expression. Examination of gene expression and fruiting body morphogenesis in signalling-deficient mutants has shown that the A and B signals become important early on in development, the D and E signals become important for development after 3–5 h, and the C signal becomes important for development after about 6 h.

Recent findings indicate that M. xanthus uses a wide array of different gene-regulatory proteins to direct developmental gene expression. These proteins include alternative sigma-factors (Apelian & Inouye, 1990, 1993; Ueki & Inouye, 1998, 2001), DNA-binding response regulators (Ellehauge et al., 1998; Ogawa et al., 1998, 2003), and transcriptional regulators.

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### METHODS

**Growth, development, motility assays, extracellular complementation and measurements of β-galactosidase activity.** *Escherichia coli* strains were grown in LB broth in the presence of relevant antibiotics (Sambrook et al., 1989). M. xanthus cells were grown in CTT medium in liquid cultures or on CTT agar plates (Hodgkin & Kaiser, 1977). Kanamycin or oxytetracycline were used for selective growth at concentrations of 40 µg ml⁻¹ and 10 µg ml⁻¹, respectively. Aggregation was monitored on CF agar (Shimkets & Kaiser, 1982) as described previously (Søgaard-Andersen et al., 1996). Briefly, cells were grown to a density of 5 × 10⁹ cells ml⁻¹ in CTT, harvested and resuspended in TPM buffer (10 mM Tris/HCl pH 7.6, 1 mM KH₂PO₄, 8 mM MgSO₄) at a calculated density of 5 × 10⁸ cells ml⁻¹. Aliquots (20 µl) of concentrated cells were spotted on CF agar and incubated at 32 °C. Aggregation was followed visually using a Leica MZ8 stereomicroscope. Cells were photographed using a Sony 3CCD camera. Levels of sporulation were determined after development for 72 h and 120 h on CF agar. Spore titres were determined as the number of sonication- and heat-resistant cfu (Søgaard-Andersen et al., 1996). In co-development experiments, cells were induced to develop on CF agar. Cells were harvested at the indicated time points and specific activities of β-galactosidase quantified (Kroos et al., 1986). Strains to be tested for motility were grown in CTT to a density of 5 × 10⁹ cells ml⁻¹, harvested and resuspended in TPM buffer to a calculated density of 5 × 10⁸ cells ml⁻¹. Five microlitres of cell suspension was spotted on a thin layer of 1:5% agar supplemented with 0.5% CTT prepared on a sterile microscope slide. The swarm edge morphology was inspected after 24 h (Hodgkin & Kaiser, 1979b).

**Bacterial strains and plasmids.** M. xanthus strains used in this work are listed in Table 1. SA1310, SA1623, SA1626, SA1627 and SA1628 were constructed by generalized transduction using Mx4 propagated on DK162200021, DK162202525, DK162204074,

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<th>Strain or plasmid</th>
<th>Genotype or description</th>
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<td><em>Ts5lac Ω4401</em></td>
<td>Kroos et al. (1986)</td>
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<tr>
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<td>Stratagene</td>
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<td>pBGS18</td>
<td>Cloning vector, Kan′</td>
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<tr>
<td>pMN306</td>
<td><em>M. xanthus</em> genomic DNA from −1500 to 2668 in pBGS18</td>
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<tr>
<td>pMN308</td>
<td><em>M. xanthus</em> genomic DNA from +1545 to +1948 in pBGS18</td>
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DK1622d4409 and DK1622d4695, respectively, to infect DK1622. SA1311, SA1312, SA1313, SA1315, SA1316, SA1317, SA1319, SA1323, SA1324 and SA1326 were constructed by generalized transduction using Mxi4 propagated on SA1310 to infect DK1217, DK1300, SA1704, DK4293, DK4300, DK4368, DK4499, DK5279, DK9007 and DK11063, respectively. All strains constructed by generalized transduction were tested by Southern blot analyses (Sambrook et al., 1989). SA1332, SA1333 and SA1335 were constructed by homologous integration of plasmid pMN304, pMN306 and pMN308, respectively, into the chromosome after electroporation (Kashefi & Hartzell, 1995). Integration was verified by Southern blot analyses (Sambrook et al., 1989). Plasmids used in this work are listed in Table 1. pMN300 construction. A 1691 bp XhoI fragment containing miniTn5(tet) g002021 and flanking DNA sequences was cloned in the XhoI site in pBluescriptIISK(−) (Stratagene). This fragment contains M. xanthus genomic DNA from −1896 to +1708 (all coordinates are relative to the start codon in hthA). pAAR106 construction. A 6732 bp XhoI fragment containing miniTn5(tet) ΔI02525 and flanking DNA sequences was cloned in the XhoI site in pBluescriptIISK(−). This fragment contains M. xanthus genomic DNA from −1896 to +1708. pMN301 construction. From pMN300 a 1183 bp PstI–XhoI fragment extending from +524 to +1708 was cloned in pBluescriptIISK(−). pMN302 construction. From pAAR106 a 2025 bp SacI–PstI fragment extending from −1500 to +524 was cloned in the same sites in pMN301. pMN302 contains M. xanthus genomic DNA from −1500 to +1708. pMN303 construction. From pMN302 a 3205 bp SacI–XhoI fragment extending from −1500 to +1708 was cloned in the same sites in pBSG18 (Spratt et al., 1986). pMN306 construction. A 1691 AatII–SalI fragment extending from position +978 to +2668 was generated by PCR using chromosomal DNA from DK1622 as a template and the primers hth7 and hth8 (primers used in this work are listed in Table 2) followed by restriction with AatII and SalI. This fragment was cloned in the same sites as in pMN302. From this pMN302 derivative a 4169 bp SacI–SalI fragment was cloned in the same sites in pBSG18 to generate pMN306. pMN306 contains M. xanthus genomic DNA from −1500 to +2668. pMN308 construction. A 404 bp blunt-end fragment extending from +1545 to +1948 was generated by PCR using chromosomal DNA from DK1622 as a template and the primers hth3 and hth4 (see Table 2) followed by treatment with T4 DNA polymerase (New England Biolabs). This fragment was cloned in the Smal site in pBSG18 to generate pMN308. All plasmids generated by PCR were verified by sequence analyses. Plasmids were propagated in TOP10 [F’ mcrA Δ(mcrA-hsdRMS-mcrBC) ΔGalK ΔlacZAM15 ΔaraX74 ΔdeoR recA1 araD139 Δ(lara-leu)7679 galU galK rpsL endA1 napG] (Invitrogen).

**DNA sequencing of chromosomal DNA flanking miniTn5(tet) insertions.** The chromosomal DNA flanking the miniTn5(tet) insertions was sequenced using arbitrary PCR (Rasmussen & Søgaard-Andersen, 2003). The sequence from −1896 to +2668 was obtained as follows. pMN300 and pAAR106 were opened with a restriction enzyme with a unique restriction site in the M. xanthus sequence and exposed to ExoSizeIII digestion (New England Biolabs). The plasmids were religated and sequenced with primers that anneal to the multiple cloning sites in pBluescriptIISK(−). Sequencing with specific primers closed any gaps in the sequence. Subsequently, this sequence was used to retrieve approximately 4000 bp on both sides of the insertions from the genome database provided by the Monsanto Company.

**RT-PCR.** To perform non-quantitative RT-PCR, RNA was isolated from vegetative cells. RNA was extracted by the hot-phenol method (Sambrook et al., 1989). The RNA was DNase I treated and re-extracted by phenol/chloroform extraction. Each of the primers hth2, hth4 and hth6 was used in a reverse transcription reaction with 100 ng total RNA using AMV RT (Finnzymes) according to the manufacturer’s recommendations. Subsequently, the primer pairs hth1-2, hth3-4 and hth5-6 were used in the PCR reaction with Taq polymerase (Promega) according to the manufacturer’s recommendations. To perform quantitative RT-PCR, cells were developed on CF agar for the indicated periods of time and harvested. RNA was extracted as described above. The RNA was reverse-transcribed using TaqMan Reverse Transcription Reagents with the supplied hexamers according to the protocol recommended by the supplier (Applied Biosystems). cDNA was purified using High Pure PCR Product Purification Kit (Roche). SYBR Green PCR Master Mix was added to cDNA from the reverse transcription of 100 ng RNA together with 500 nM of each of the two primers, hthF and hthR. The primers hybridize to the middle of hthA and give rise to a PCR product with a size of 65 bp (see Table 2). The RT-PCR reaction was performed on an ABI PRISM 7700 Sequence Detection System (Applied Biosystems) using the standard set-up. Primers were designed using PrimerExpress as recommended by the ABI PRISM 7700 Sequence Detection System supplier. The level of hthAB transcript detected is expressed as relative units per ng total RNA.

**RESULTS**

A new genetic locus required for fruiting body morphogenesis

We previously reported the isolation of a collection of M. xanthus mutants, which display abnormal fruiting body morphogenesis (Rasmussen & Søgaard-Andersen, 2003). The sequence from −1896 to +2668 was obtained as follows. pMN300 and pAAR106 were opened with a restriction enzyme with a unique restriction site in the M. xanthus sequence and exposed to ExoSizeIII digestion (New England Biolabs). The plasmids were religated and sequenced with primers that anneal to the multiple cloning sites in pBluescriptIISK(−). Sequencing with specific primers closed any gaps in the sequence. Subsequently, this sequence was used to retrieve approximately 4000 bp on both sides of the insertions from the genome database provided by the Monsanto Company.

**Table 2. Primers**

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<td>+697 to +715</td>
<td>CGCCGCCGCCTGGATTGTC</td>
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<td>+1020 to +1002</td>
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</tr>
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<td>+1545 to +1568</td>
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<td>hthR</td>
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2003). This collection of mutants was isolated after mutagenesis of the fully motile strain DK1622, which serves as the wild-type strain in this study, with the miniTn\(^5\)\((tet)\) transposon. Five of these mutants (DK1622-V\(0021\), -V\(2525\), -V\(4074\), -V\(4409\) and -V\(4695\)) showed similar developmental defects, displaying delayed aggregation and reduced sporulation on CF starvation medium (data not shown). Sequence determination using arbitrary PCR of the regions flanking the five miniTn\(^5\)\((tet)\) insertions showed that they were all inserted into the same gene (see below). To ensure that the developmental phenotype in these DK1622 derivatives was caused by the miniTn\(^5\)\((tet)\) insertions, the insertions were crossed back into the wild-type strain DK1622 to generate SA1310 and SA1625–SA1628 by Mx4-dependent generalized transduction. The developmental phenotype of the five mutant strains was tested on CF starvation medium. Like the five parent strains, the five back-crossed strains displayed similar phenotypes in which aggregation was delayed and mound formation was not evident until 72 h (Fig. 1; note that only the phenotype of SA1310, which carries miniTn\(^5\)(tet) \(\Omega\)0021, is shown), whereas mound formation in DK1622 was evident after 24 h. Moreover, the mounds formed by the five mutants strains were smaller than those formed by DK1622, and the five mutant strains formed approximately twice as many mounds as DK1622 (Fig. 1). The effect of the miniTn\(^5\)\((tet)\) \(\Omega\)0021, \(\Omega\)2525, \(\Omega\)4074, \(\Omega\)4409 and \(\Omega\)4695 insertions on sporulation was assessed after starvation on CF medium. All five mutant strains behaved similarly (Table 3; note that only the sporulation data for SA1310, which carries miniTn\(^5\)(tet) \(\Omega\)0021, are shown). At 72 h, SA1310 sporulated at a level 400-fold lower than that of DK1622; after 120 h, the sporulation level in SA1310 was fivefold lower than in DK1622. As the phenotype of the five backcrossed strains

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**Table 3. Sporulation frequencies of wild-type and mutant M. xanthus strains**

Cells were starved on CF starvation medium for the indicated periods of time. Sporulation frequencies are presented relative to the level of sporulation in DK1622 after 120 h as means ± SD from three experiments. Spore titres were determined as the numbers of sonication- and heat-resistant c.f.u. (Søgaard-Andersen et al., 1996). The absolute sporulation frequency of DK1622 after 120 h was 4 %.

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<td></td>
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<td>72 h</td>
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<tr>
<td>DK1622</td>
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<td>SA1310</td>
<td>(hthA::\text{miniTn}^5(tet)) (\Omega)0021</td>
<td>30 ± 8</td>
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<td>SA1332</td>
<td>(hthA::\text{miniTn}^5(tet)) (\Omega)0021/pMN304</td>
<td>0-08 ± 0-03</td>
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<td>(hthA::\text{miniTn}^5(tet)) (\Omega)0021/pMN306</td>
<td>0-10 ± 0-03</td>
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<tr>
<td>SA1335</td>
<td>(hthB::\text{pMN308})</td>
<td>38 ± 10</td>
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<tr>
<td></td>
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<td>0-05 ± 0-03</td>
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is similar to that of the five original mutants, these data suggest that the miniTn5(tet) insertions are responsible for the mutant phenotype. From here on, only data for the mutant strain, which carries miniTn5(tet) Ω0021, are shown.

Cells of SA1310 appeared indistinguishable from wild-type cells during vegetative growth with respect to growth rate in CTT medium and A1 minimal medium, colony morphology on CTT agar, and pigmentation (data not shown).

Two genetic systems control gliding motility in *M. xanthus* (Spormann, 1999). The social (S)-motility system controls gliding of groups of cells, and the adventurous (A)-motility system controls gliding of single cells. To test the effect of miniTn5(tet) Ω0021 on motility, the insertion was transduced into representative S− (DK1300 sglG1) and A− (DK1217 aglB1) backgrounds by Mx4-dependent generalized transduction. Strains that carry both an A− mutation and an S− mutation are non-swarming and grow as small, smooth-edged colonies (Hodgkin & Kaiser, 1979a, b). The miniTn5(tet) Ω0021 insertion did not generate a non-swarming phenotype when crossed into A− or S− mutant backgrounds (Fig. 2). Thus, the miniTn5(tet) Ω0021 insertion does not interfere with either the A- or the S-motility system in vegetative cells, suggesting that the gene carrying the miniTn5(tet) Ω0021 insertion is not a constituent of either the A- or the S-motility system. Together, these data provide evidence that the developmental defects caused by the miniTn5(tet) Ω0021 insertion are not secondary to an effect on motility.

**Fig. 2.** Effect of an hthA*hthB* mutation on vegetative motility. Cells of the various strains were spotted on 1·5% CTT and viewed in a Leica MZ8 stereomicroscope. Swarm edges were recorded after 24 h of incubation at 32 °C. Strains in the left vertical column are all *hthA*+*hthB*+; strains in the right vertical column carry miniTn5(tet) Ω0021 and are all *hthA*hthB*. Strains in the left column are from top to bottom: DK1622 (wild-type), DK1300 (sglG1), DK1217 (aglB1). Strains in the right vertical column are from top to bottom: SA1310 (*hthA::miniTn5(tet) Ω0021*), SA1312(sglG1, *hthA::miniTn5(tet) Ω0021*) and SA1311 (aglB1, *hthA::miniTn5(tet) Ω0021*). Bar, 0·5 mm.

**Fig. 3.** Analysis of the *hthAB* region. (a) Open reading frames are indicated by open arrows. Arrows indicate the direction of transcription. Coordinates are relative to +1, the first nucleotide in the start codon of *hthA*. Below the physical map, the overlap between the *hthA* stop codon and *hthB* start codon is shown. The lollipops indicate the sites of insertion of miniTn5(tet) in *hthA*. miniTn5(tet) Ω0021, Ω2525, Ω4074, Ω4409 and Ω4695 are inserted at position +107, +1093, +147, +538 and +559, respectively. Plasmids containing the indicated DNA fragments of the *hth* operon are shown below the map. (b) Non-quantitative RT-PCR analysis of the *hth* operon structure. Total RNA was isolated from vegetative DK1622 (wild-type) cells (lanes 1, 3 and 5) and SA1310 (miniTn5(tet) Ω0021) cells (lanes 2, 4 and 6). Primers used in RT-PCR reactions are indicated above the lanes: lane 1 and 2, *hth5* and *hth6* (*hth5* hybridizes to *hthA* and *hth6* hybridizes to *hthB*; see Table 2); lanes 3 and 4, *hth1* and *hth2* (both hybridize to *hthA*; see Table 2); lanes 5 and 6, *hth3* and *hth4* (both hybridize to *hthB*; see Table 2).

**Analysis of the hth locus**

Analyses of the DNA sequences surrounding the insertion sites of miniTn5(tet) Ω0021, Ω2525, Ω4074, Ω4409 and Ω4695 revealed the presence of four ORFs (Fig. 3a). These ORFs were generally identified on the basis of a high GC content in the third positions of codons typical of GC-rich organisms (Bibb *et al.*, 1984; Shimkets, 1990). miniTn5(tet) Ω0021 as well as Ω2525, Ω4074, Ω4409 and Ω4695 are inserted in an 1128 bp ORF, which has previously been labelled *hthA* (GenBank accession no. AF375047) (Fig. 3a). However, the function of *hthA* in *M. xanthus* has not been described. Immediately downstream from *hthA*, an 1170 bp
ORF, which we denote hthB, is located. hthB is transcribed in the same direction as hthA and the stop codon of hthA overlaps partially with the putative start codon of hthB (Fig. 3a). In a region covering 710 bp upstream from hthA no ORFs were identified. Upstream from this region a 1593 bp ORF, which we denote orfA and which is transcribed in the same direction as hthA, is located. Downstream from hthB a 1356 bp ORF, which we denote orfB and which is transcribed in a direction opposite to that of hthA, is located. The last 29 bp of orfB overlap with the last 29 bp of hthB (Fig. 3a).

The deduced hthA gene product contains 375 amino acids. Sequence analyses (Altschul et al., 1990) indicates that the C-terminal part of HthA (amino acids 318–375) contains a helix–turn–helix DNA-binding domain, which is found in a large number of DNA-binding proteins, for example the FixL subfamily of DNA-binding response regulators (Parkinson & Kofoid, 1992), the LuxR family of autoinducer binding transcriptional regulators (Fuqua et al., 2001), the small DNA-binding protein GerE from Bacillus subtilis (Zheng et al., 1992) and MalT from E. coli (Cole & Raibaud, 1986) (Fig. 4). To further assess the possibility that HthA contains a C-terminal helix–turn–helix DNA-binding domain, the C-terminal 58 amino acids in HthA were compared to the DNA-binding domain of NarL, which is a FixL-type response regulator. The DNA-binding domain in NarL consists of a four α-helix bundle (Baikalov et al., 1996). A hydrophobic core and a salt bridge maintain the tertiary structure of this domain. The residues involved in maintaining the hydrophobic core and in establishing the salt bridge are conserved in the C-terminal part of HthA (Fig. 4), supporting the notion that this part of HthA constitutes a DNA-binding domain. Interestingly, the deduced amino acid sequence of the N-terminal part of HthA yielded no significant similarities in BLAST searches. From these analyses, we suggest that HthA is a DNA-binding protein.

The deduced hthB and orfA gene products contain 389 and 530 amino acids, respectively. Sequence analyses of HthB and OrfA revealed that none of these two proteins share significant homology to proteins in the databases. The deduced orfB gene product contains 451 amino acids. Sequence analyses showed that OrfB contains a thioredoxin domain from amino acid residue 49 to 118. The remainder of the OrfB protein does not share significant homology to proteins in the databases.

**hthA and hthB are co-transcribed**

The overlap between the stop codon of hthA and the start codon of hthB suggested that these two genes are cotranscribed. To test this idea, non-quantitative RT-PCR analyses were carried out on total RNA isolated from vegetative wild-type cells using primer pairs specific for hthA and hthB, respectively, and a primer pair in which one primer hybridized to hthA and the second primer hybridized to hthB. All three primer pairs gave rise to a PCR product with the correct size in the RT-PCR reactions (Fig. 3b), thus suggesting that hthA and hthB constitute an operon.

Insertion of miniTn5(tet) into a gene is likely to inactivate it. Moreover, miniTn5(tet) may have polar effects on downstream genes. Thus, in the case of miniTn5(tet) Ω0021, the insertion may inactivate hthA and have a polar effect on hthB. To determine whether miniTn5(tet) Ω0021 has a polar effect on hthB transcription, RT-PCR analyses were carried out on total RNA isolated from vegetative SA1310 cells using the same three primer pairs as above. As shown in Fig. 3(b), no PCR products were detected in the RT-PCR reactions in these experiments. Thus, miniTn5(tet) Ω0021 insertion has a polar effect on hthB transcription.

**Expression of the hthAB genes during development**

To study the expression of the hthAB genes, quantitative RT-PCR analyses were performed on total RNA isolated from vegetative and starving DK1622 cells. The relative levels of hth mRNA decreased 500-fold between 0 and 6 h of starvation. After 12 h, the hth mRNA was not detectable in the RT-PCR analyses. As a control, the expression of the todK gene was also analysed by quantitative RT-PCR.

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**Fig. 4.** Alignment of the C-terminal part of HthA with the DNA-binding domains in NarL from E. coli (CAA48935), DegU from B. subtilis (RGSB9D), FixJ from R. meliloti (P10958), UhpA from E. coli (P10940), GerE from B. subtilis (P11470), LuxR from V. fischeri (P12748), TraR from A. tumefaciens (AAC17192) and MalT from E. coli (P06993). The helix–turn–helix motif in NarL is indicated. Asterisks indicate residues involved in maintaining the tertiary structure of the DNA-binding domain in NarL. Residues in black, dark grey and light grey backgrounds are conserved 100%, 75% and 50%, respectively. The alignment was made using CLUSTALX (Thompson et al., 1997); the presentation was made using Genedoc (http://www.psc.edu/biomed/genedoc).
As previously reported (Rasmussen & Søgaard-Andersen, 2003), expression of todK was observed to decrease 10-fold during the first 12 h of starvation. Thus, transcription of the hthAB genes decreases strongly in response to starvation.

**Identification of the genes in the hth locus required for aggregation and sporulation**

To determine whether the defects caused by the miniTn5(tet) Ω0021 insertion were due to loss of hthA and/or hthB function, genetic complementation experiments were performed. The plasmid pMN304 carries the wild-type hthA gene including 1500 bp upstream from the putative start codon of hthA (Fig. 3a). The plasmid pMN306 carries the wild-type hthA and hthB genes including 1500 bp upstream from the putative start codon of hthB (Fig. 3a). pMN304 and pMN306 were introduced by electroporation into SA1310, which carries the miniTn5(tet) Ω0021 insertion, to give strains SA1332 and SA1333, respectively. In both strains, the plasmid had integrated by homologous recombination upstream from the miniTn5(tet) Ω0021 insertion. SA1332 carries an intact hthA gene including the native promoter upstream from the integrated plasmid and the 3'-end of orfA, hthA::miniTn5(tet) Ω0021 and hthB downstream from the integrated plasmid. SA1333 carries intact copies of hthA and hthB including the native promoter upstream from the integrated plasmid and the 3'-end of orfA, hthA::miniTn5(tet) Ω0021 and hthB downstream from the integrated plasmid. SA1332 and SA1333 were assayed for development on CF starvation medium in parallel with the wild-type strain DK1622 and SA1310. As shown in Fig. 1 and Table 3, the aggregation defect caused by the miniTn5(tet) Ω0021 insertion was corrected by pMN304 in SA1332. However, pMN304 did not correct the sporulation defect caused by miniTn5(tet) Ω0021. On the other hand, both the aggregation defect and the sporulation defect caused by miniTn5(tet) Ω0021 were corrected by pMN306 in SA1333. These observations show that the developmental defects caused by miniTn5(tet) Ω0021 are due to a loss of both hthA and hthB function. Moreover, these data provide evidence that in a strain lacking both HthA and HthB, complementation with hthA restores the aggregation defect whereas hthB is required to restore the sporulation defect.

The results of the genetic complementation analyses suggested that HthB is only required for sporulation. To test this hypothesis, a strain that carries an hthB mutation was constructed. The plasmid pMN308 was constructed by cloning a 404 bp DNA fragment, which is located within hthB and extends from position +1545 to +1948 in hthB (Fig. 3a), in the plasmid pBG518 that confers resistance to kanamycin. pMN308 was introduced into the wild-type DK1622 by electroporation to give strain SA1335. In SA1335, pMN308 has integrated by homologous recombination as a result of single crossover in the hthB gene. A single crossover yields kanamycin-resistant electroporants with two incomplete copies of hthB and is, therefore, likely to inactivate the hthB gene. To examine the possible developmental defects caused by the hthB::miniTn5 mutation, SA1335 was exposed to starvation on CF starvation medium in parallel with the wild-type strain DK1622 and SA1310, which carries miniTn5(tet) Ω0021. SA1335 had an aggregation phenotype similar to that of DK1622, and thus displayed no aggregation defects (Fig. 1). However, the level of sporulation in SA1335 was reduced compared to that in DK1622 and similar to that of SA1310 after 72 h and 120 h. Taken together, these data show that loss of hthB function results in a sporulation defect.

**Loss of hthA and hthB function does not interfere with synthesis of intercellular signals**

To investigate whether the sporulation defect caused by miniTn5(tet) Ω0021 was cell-autonomous, cells of SA1310 were co-developed with an equal number of wild-type cells

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**Table 4. Sporulation frequencies of mutant M. xanthus strains in co-development experiments**

Cells were starved on CF starvation medium for 72 h. Sporulation frequencies are presented relative to the level of sporulation in DK1622 after 120 h as means ± SD from three experiments. Spore titres were determined as the numbers of sonication- and heat-resistant c.f.u. (Søgaard-Andersen et al., 1996). The absolute sporulation frequency of DK1622 after 120 h was 4%. NA, Not applicable.

<table>
<thead>
<tr>
<th>Test strain (genotype)</th>
<th>Sporulation frequency of test strain during co-development with:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No other strain</td>
</tr>
<tr>
<td>SA1310 (hthA::miniTn5(tet) Ω0021)</td>
<td>0.08 ± 0.03</td>
</tr>
<tr>
<td>DK4398 (agsB, Tn5lac Ø4411)</td>
<td>5.5 ± 1.2</td>
</tr>
<tr>
<td>DK11015 (bsgA::Tn5 lac Ø7516)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>LS269 (csgA::Tn5lac Ø269)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>DKJ260 (dsag29 Tn5 Ø1867)</td>
<td>&lt;0.0001</td>
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<tr>
<td>JD300 (esg::Tn5 Ø258)</td>
<td>&lt;0.0001</td>
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</table>
and the number of spores formed by the SA1310 cells was measured after 72 h of starvation. The sporulation defect in SA1310 remained unaffected by co-development with wild-type cells (Table 4). Moreover, we found that SA1310 cells rescued sporulation in mutants lacking the A, B, C, D or E signal as efficiently as wild-type cells by extracellular complementation (Table 4). Taken together, these observations suggest that the developmental defects caused by miniTn5(tet) 0021 are cell-autonomous and that miniTn5(tet) 0021 does not interfere with the synthesis of intercellular signals required for fruiting body morphogenesis.

Effect of loss of hthA and hthB function on developmental gene expression

To further define the role of HthA and HthB, the expression of eight developmentally regulated lacZ reporter fusions was studied in the miniTn5(tet) 0021 mutant background. In wild-type cells, each fusion increases its expression at a particular time point during starvation. The eight fusions have different expression times ranging from 0 to 24 h. Thus, analysis of the expression of these reporter fusions in the hthAB mutant allows us to monitor the progression of the developmental programme in the mutant. To analyse the effect of the miniTn5(tet) 0021 insertion on developmental gene expression, the mutation was transduced into the eight strains, each containing a reporter fusion. Subsequently, isogenic hthA\textsuperscript{+}hthB\textsuperscript{+} and hthAhthB strains each carrying one of the lacZ fusions were exposed to starvation on CF starvation medium and specific activities of β-galactosidase expressed from the fusions determined (Fig. 5). The expression profiles of Tn5lac 44521, csgA–lacZ (pJM200), Tn5lac 44499, Tn5lac 44403 and Tn5lac 44401 were indistinguishable in the two strains. The three fusions Tn5lac 4408, Tn5lac 47540 and Tn5lac 4414 had altered expression profiles in the hthAhthB strain. In the case of Tn5lac 4408, the expression profile in the hthAhthB strain was similar to that in the wild-type; however, the level of expression was higher in the hthAhthB strain at all time points including 0 h of starvation, i.e. in non-starved cells, and, after 24 h, the level of expression was approximately 2.5-fold higher in the hthAhthB strain than in the wild-type. In the case of Tn5lac 47540, the level of expression was similar in both strains until 3 h; from then on, the level of expression in the hthAhthB strain was lower than in the wild-type and, after 24 h, the level of β-galactosidase expression was approximately 2.5-fold lower in the hthAhthB strain than in the wild-type. In the case of Tn5lac 4414, the level of expression was similar in both strains until 6 h; from then on, the level of expression in the hthAhthB strain was lower than in the wild-type and, after 48 h, the level of β-galactosidase expression was approximately sixfold lower in the hthAhthB strain than in the wild-type. From these analyses, it is apparent that hthA and hthB are important for developmental gene expression and that loss of function of the two proteins results in pleiotropic effects on developmental gene expression.

![Fig. 5. Effect of hthAhthB mutation on developmental gene expression. Expression of the indicated lacZ fusions in wild-type DK1622 cells (■) and in hthAhthB SA1310 cells (○). Cells were starved on CF agar, samples withdrawn at the indicated time points and specific activities of β-galactosidase determined. The experiments were done in triplicate. Error bars indicate standard deviations. Specific activities of β-galactosidase are given in nmol o-nitrophenol min\textsuperscript{-1} (mg protein\textsuperscript{-1}).](http://mic.sgmjournals.org)
DISCUSSION

Aggregation and sporulation are the two major morpho-genetic events underlying fruiting body formation in *M. xanthus*. Here we report the identification of two genes, *hthA* and *hthB*, which are important for aggregation and sporulation. Loss of HthA and HthB function results in delayed aggregation and in the formation of small-sized fruiting bodies. Extracellular complementation experiments provided evidence that the developmental defects caused by loss of HthA and HthB function are cell-autonomous. Consistently, loss of HthA and HthB function does not interfere with the synthesis of intercellular signals required for fruiting body morphogenesis. Genetic complementation of an *hthAB* mutant with a plasmid carrying *hthA* restores the aggregation defect, whereas the sporulation defect is not corrected. However, genetic complementation of the *hthAB* mutant with a plasmid carrying *hthA* as well as *hthB* restores the aggregation defect as well as the sporulation defect. Specific inactivation of *hthB* results in delayed and reduced sporulation. These results provide evidence that HthA, independent of HthB, functions to promote the aggregation process. Moreover, the data provide evidence that HthB functions to promote the sporulation process. However, it remains to be clarified whether HthA and HthB interact to promote sporulation, i.e. HthB could act independently of HthA during sporulation or HthA and HthB may interact during the sporulation process.

Analysis of the primary sequence of the C-terminal part of HthA showed that this part of HthA has similarity to the DNA-binding domain found in several DNA-binding proteins. Moreover, a detailed comparison of the C-terminal part of HthA to the DNA-binding domain in NarL revealed that the amino acid residues involved in maintaining the tertiary structure of the DNA-binding domain in NarL are conserved in HthA. Thus, at the structural level, HthA is predicted to contain a C-terminal helix–turn–helix DNA-binding motif.

The activity of transcriptional regulators containing the DNA-binding domain found in HthA is modulated in distinct ways: the activity of the FixJ-like DNA-binding response regulators is modulated by phosphorylation of a conserved Asp residue in the N-terminal receiver part of response regulators is modulated by phosphorylation of a conserved Asp residue in the N-terminal receiver part of response regulators (Ducros et al., 2001); the GerE protein only consists of the four α-helix bundle DNA-binding domain and the activity is not modulated by either covalent modification or ligand binding (Ducros et al., 2001); finally, the MalT protein in *E. coli* is activated by binding of ATP and maltotriose (Raibaud & Richet, 1987; Richet & Raibaud, 1989). Intriguingly, the N-terminal part of HthA does not exhibit significant similarity to any proteins in the databases. This lack of homology raises the possibility that HthA contains a novel domain involved in modulating transcriptional activity. Alternatively, this part of HthA could be involved in oligomerization, as has been found in TraR (Zhang et al., 2002). In conclusion, the data support the notion that HthA contains a C-terminal DNA-binding domain and may act as a transcriptional regulator. This hypothesis is supported by the observation that loss of HthA and HthB function alters the gene expression profile in vegetative as well as in starving cells (see below).

The primary sequence of the HthB protein does not share significant homology to proteins in the databases. Therefore, the mode of action of the HthB protein in the sporulation process remains unknown. Likewise, our data do not allow us to conclude whether HthB functions independently of HthA during sporulation. In principle, HthA and HthB could act independently during fruiting body formation, with HthA being important for aggregation and HthB being important for sporulation. HthA and HthB could also interact directly or indirectly to promote sporulation. Given that the activity of some of the transcriptional regulators containing the DNA-binding domain found in HthA is regulated by ligand binding, an interesting possibility for an indirect interaction between HthA and HthB could be that HthB possesses enzymic activity and is involved in the synthesis of an HthA ligand, which is required for full activity of HthA. Alternatively, HthB may be required for full expression of *hthA*. Further experiments are needed to discriminate between these possibilities.

RT-PCR analyses provided evidence that the *hthA* and *hthB* genes are co-transcribed. No ORFs were identified in the 710 bp between the stop codon in *orfA* and the start codon of *hthA* (Fig. 3), suggesting that *hthA* and *hthB* may constitute a two-gene operon. *hthA* and *hthB* are expressed in vegetative cells. Accumulation of the *hthAB* mRNA decreases approximately 500-fold within the first 6 h of starvation, and after 12 h, the *hthAB* mRNA is no longer detectable. Assuming that the intracellular concentration of the HthA and HthB proteins follows the detection profile of *hthAB* mRNA, then HthA and HthB are present in vegetative cells and the cellular concentration of the two proteins decreases in response to starvation. Consistent with the notion that HthA and HthB are present in vegetative cells, an *hthAhthB* mutation results in increased transcription of the transcriptional *sdeK–lacZ* fusion (Tn5lac Ω4408) in vegetative cells (see below).

Loss of HthA and HthB function has pleiotropic effects on gene expression. Loss of HthA and HthB function results in increased expression of a transcriptional *sdeK–lacZ* fusion (Tn5lac Ω4408) in non-starving cells and during development. SdeK encodes a histidine protein kinase important for fruiting body formation (Garza et al., 1998; Pollock & Singer, 2001) and, normally, expression of *sdeK* is activated early during development in a stringent-response-dependent manner from a putative sigma-54-dependent promoter (Garza et al., 1998; Singer & Kaiser, 1995). The effect of loss of HthA and HthB function on *sdeK* expression provides evidence that HthA and/or HthB – directly or indirectly – inhibit transcription of *sdeK*
in vegetative cells and in developing cells. Loss of HthA and HthB function also results in decreased levels of expression of transcriptional fruA–lacZ (Tn5lac Ω7540) and devR–lacZ fusions (Tn5lac Ω4414) during development. fruA is normally expressed after 3–6 h of starvation and encodes a DNA-binding response regulator, which is a key component in the C-signal transduction pathway (Ellehauge et al., 1998; Horiuchi et al., 2002; Ogawa et al., 1996; Søgaard-Andersen & Kaiser, 1996). devR is normally expressed after 6–9 h of starvation and encodes a protein of unknown function, which is important for sporulation (Thöny-Meyer & Kaiser, 1993). These data argue that HthA and/or HthB – directly or indirectly – stimulate transcription of fruA and devR. Loss of SdeK function does not change expression of fruA (A. Aa. Rasmussen, unpublished), suggesting that the decreased transcription of fruA in the hthAhthB mutant is not caused by the increased levels of SdeK. On the other hand, both SdeK and FruA stimulate transcription of the devR–lacZ fusion (Tn5lac Ω4414) (Ellehauge et al., 1998; Kroos et al., 1990) and Tn5lac Ω4403 (E. Ellehaug, unpublished; Pollack & Singer, 2001). Therefore, the expression profile of the devR–lacZ fusion (Tn5lac Ω4414) in the hthAhthB mutant could be explained by the lack of FruA protein being dominant over the increased level of SdeK. The observation that the expression profile of Tn5lac Ω4403 in the hthAhthB mutant is similar to the expression profile in wild-type cells could be explained by the increase in SdeK and decrease in FruA levels balancing each other.

How does loss of HthA and HthB function result in developmental defects? Complete loss of FruA function results in aggregation and sporulation defects (Ellehauge et al., 1998; Ogawa et al., 1996). Likewise, loss of devR function results in a sporulation defect (Kroos et al., 1990). We speculate that the decreased expression of fruA and devR contributes to the aggregation and sporulation defects in the hthAB mutant. Based on the strong decrease in transcription of the hthAB genes in response to starvation it is tempting to speculate that HthA and/or HthB may have their primary function in vegetative cells and only directly regulate gene expression in vegetative cells. In this scenario, proteins encoded by those genes that are regulated by HthA and/or HthB in vegetative cells would, in turn, be involved in the expression of genes important for aggregation and sporulation. Specifically, genes expressed in an HthA-dependent manner in vegetative cells would direct the expression of genes important for aggregation. Similarly, genes expressed in an HthB-dependent manner in vegetative cells would direct the expression of genes important for sporulation. In ongoing experiments we are addressing the identification of genes that are directly regulated by HthA and/or HthB.

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