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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.

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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

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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.*, 1996) including several NtrC-like activators (Caberoy *et al.*, 2003; Gronewold & Kaiser, 2001, 2002; Guo *et al.*, 2000; Hager *et al.*, 2001; Sun & Shi, 2001a, b), MrpC, a homologue of the *E. coli* CRP protein (Sun & Shi, 2001a), and AsgB, which contains a C-terminal DNA-binding domain and a novel oligomerization and/or regulatory domain (Plamann *et al.*, 1994). Likewise, *cis*-acting regulatory sequences important for developmental gene expression are being revealed (Brandner & Kroos, 1998; Fisseha *et al.*, 1996, 1999; Gulati *et al.*, 1995; Hao *et al.*, 2002; Keseler & Kaiser, 1995; Li *et al.*, 1992; Romeo & Zusman, 1991; Ueki & Inouye, 2003; Viswanathan & Kroos, 2003; Yoder & Kroos, 2004). Currently, the best-understood example of developmental gene expression at the molecular level involves the MrpC-dependent activation of the *fruA* gene, which encodes a DNA-binding response regulator that acts in the C-signal transduction pathway (Ellehauge *et al.*, 1998; Horiuchi *et al.*, 2002; Ogawa *et al.*, 1996; Søgaard-Andersen & Kaiser, 1996). MrpC binds to the *fruA* promoter and activates *fruA* transcription after 3–6 h (Ueki & Inouye, 2003). Interestingly, expression of *mrpC* is itself developmentally regulated (Sun & Shi, 2001b). These observations suggest that developmental gene expression and the progression of the developmental programme in *M. xanthus* involve a complicated network of transcriptional regulators.

To further understand the mechanisms involved in developmental gene expression and the coupling between morphogenesis and developmental gene expression, we have analysed an *M. xanthus* mutant that displays delayed aggregation, reduced sporulation and abnormal developmental gene expression. Here, we report the identification of two co-transcribed genes that are important for normal aggregation, sporulation and developmental gene expression. The upstream gene *hthA* is likely to encode a novel DNA-binding protein. The predicted *hthB* product lacks homology to proteins in the databases. HthA is important for aggregation, whereas HthB, alone or in combination with HthA, is important for sporulation.

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 5×10^8 cells ml^{-1} in CTT, harvested and resuspended in TPM buffer (10 mM Tris/HCl pH 7·6, 1 mM KH_2PO_4, 8 mM $MgSO_4$) at a calculated density of 5×10^9 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 c.f.u. (Søgaard-Andersen *et al.*, 1996). In co-development experiments, cells at a calculated density of 5×10^9 cells ml^{-1} were mixed at a 1 : 1 ratio and 20 μl aliquots of the mixtures were spotted on CF agar and incubated at 32 °C. To measure specific activities of β-galactosidase during development, 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 5×10^8 cells ml^{-1}, harvested and resuspended in TPM buffer to a calculated density of 5×10^9 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, SA1625, SA1626, SA1627 and SA1628 were constructed by generalized transduction using Mx4 propagated on DK1622Ω0021, DK1622Ω2525, DK1622Ω4074,

Table 1. *M. xanthus* strains and plasmids

Strain or plasmid	Genotype or description	Reference
M. xanthus		
DK1622	Wild-type	Kaiser (1979)
DK1217	*aglB1*	Hodgkin & Kaiser (1979b)
DK1300	*sglG1*	Hodgkin & Kaiser (1979b)
DK3260	(*dsg429* Tn*5* Ω1867)	Cheng & Kaiser (1989)
DK4293	Tn*5lac* Ω4401	Kroos *et al.* (1986)
DK4300	*sdeK*::Tn*5lac* Ω4408	Kroos *et al.* (1986)
DK4368	Tn*5lac* Ω4403	Kroos *et al.* (1986)
DK4398	(*asgB*, Tn*5lac* Ω4411)	Kuspa *et al.* (1986)
DK4499	Tn*5lac* Ω4499	Kroos *et al.* (1986)
DK5279	*devR*::Tn*5lac* Ω4414	Kroos *et al.* (1986)
DK9007	Tn*5lac* Ω4521	Rasmussen & Søgaard-Andersen (2003)
DK11015	(*bsgA*::Tn*5 lac* Ω7516)	Søgaard-Andersen *et al.* (1996)
DK11063	*fruA*::Tn*5lac* Ω7540	Søgaard-Andersen *et al.* (1996)
JD300	(*esg*::Tn*5* Ω258)	Toal *et al.* (1995)
LS269	(*csgA*::Tn*5lac* Ω269)	Shimkets & Asher (1988)
SA1310	*hthA*::miniTn*5*(*tet*) Ω0021	This paper
SA1311	*aglB1*, *hthA*::miniTn*5*(*tet*) Ω0021	This paper
SA1312	*sglG1*, *hthA*::miniTn*5*(*tet*) Ω0021	This paper
SA1313	$csgA^+/csgA^+$–*lacZ*, *hthA*::miniTn*5*(*tet*) Ω0021	This paper
SA1315	Tn*5lac* Ω4401, *hthA*::miniTn*5*(*tet*) Ω0021	This paper
SA1316	*sdeK*::Tn*5lac* Ω4408, *hthA*::miniTn*5*(*tet*) Ω0021	This paper
SA1317	Tn*5lac* Ω4403, *hthA*::miniTn*5*(*tet*) Ω0021	This paper
SA1319	Tn*5lac* Ω4499, *hthA*::miniTn*5*(*tet*) Ω0021	This paper
SA1323	*devR*::Tn*5lac* Ω4414, *hthA*::miniTn*5*(*tet*) Ω0021	This paper
SA1324	Tn*5lac* Ω4521, *hthA*::miniTn*5*(*tet*) Ω0021	This paper
SA1326	*fruA*::Tn*5lac* Ω7540, *hthA*::miniTn*5*(*tet*) Ω0021	This paper
SA1332	*hthA*::miniTn*5*(*tet*) Ω0021/pMN304	This paper
SA1333	*hthA*::miniTn*5*(*tet*) Ω0021/pMN306	This paper
SA1335	*hthB*::pMN308	This paper
SA1625	*hthA*::miniTn*5*(*tet*) Ω2525	This paper
SA1626	*hthA*::miniTn*5*(*tet*) Ω4074	This paper
SA1627	*hthA*::miniTn*5*(*tet*) Ω4409	This paper
SA1628	*hthA*::miniTn*5*(*tet*) Ω4695	This paper
SA1704	$csgA^+/csgA^+$–*lacZ*	Rasmussen & Søgaard-Andersen (2003)
Plasmids		
pBluescriptIISK$^-$	Cloning vector, Ap^r	Stratagene
pBGS18	Cloning vector, Kan^r	Spratt *et al.* (1986)
pMN300	*M. xanthus* genomic DNA from −1896 to +1708, miniTn*5*(*tet*) Ω0021 at +107 in pBluescriptIISK$^-$	This paper
pAAR106	*M. xanthus* genomic DNA from −1896 to +1708, miniTn*5*(*tet*) Ω2525 at +1093 in pBluescriptIISK$^-$	This paper
pMN301	*M. xanthus* genomic DNA from +524 to +1708 in pBluescriptIISK$^-$	This paper
pMN302	*M. xanthus* genomic DNA from −1500 to +1708 in pBluescriptIISK$^-$	This paper
pMN304	*M. xanthus* genomic DNA from −1500 to +1708 in pBGS18	This paper
pMN306	*M. xanthus* genomic DNA from −1500 to +2668 in pBGS18	This paper
pMN308	*M. xanthus* genomic DNA from +1545 to +1948 in pBGS18	This paper

DK1622Ω4409 and DK1622Ω4695, respectively, to infect DK1622. SA1311, SA1312, SA1313, SA1315, SA1316, SA1317, SA1319, SA1323, SA1324 and SA1326 were constructed by generalized transduction using Mx4 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 6732 bp *Xho*I fragment containing miniTn*5*(*tet*) Ω0021 and flanking DNA sequences was cloned in the *Xho*I 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 *Xho*I fragment containing miniTn*5*(*tet*) Ω2525 and flanking DNA sequences was cloned in the *Xho*I site in pBluescriptIISK$^-$. This fragment contains *M. xanthus* genomic DNA from −1896 to +1708. **pMN301 construction.** From pMN300 a 1183 bp *Pst*I–*Xho*I fragment extending from +524 to +1708 was cloned in pBluescriptIISK$^-$. **pMN302 construction.** From pAAR106 a 2025 bp *Sac*I–*Pst*I fragment extending from −1500 to +524 was cloned in the same sites in pMN301. pMN302 contains *M. xanthus* genomic DNA from −1500 to +1708. **pMN304 construction.** From pMN302 a 3205 bp *Sac*I–*Xho*I fragment extending from −1500 to +1708 was cloned in the same sites in pBGS18 (Spratt *et al.*, 1986). **pMN306 construction.** A 1691 *Aat*II–*Sal*I 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 *Aat*II and *Sal*I. This fragment was cloned in the same sites in pMN302. From this pMN302 derivative a 4169 bp *Sac*I–*Sal*I fragment was cloned in the same sites in pBGS18 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 *Sma*I site in pBGS18 to generate pMN308. All plasmids generated by PCR were verified by sequence analyses. Plasmids were propagated in TOP10 [F$^-$ *mcrA* Δ(*mrr–hsdRMS–mcrBC*) *ϕ*80*lacZ*ΔM15 Δ*lacX74 deoR recA1 araD139* Δ(*ara–leu*)*7679 galU galK rpsL endA1 nupG*] (Invitrogen).

DNA sequencing of chromosomal DNA flanking miniTn*5*(*tet*) insertions. The chromosomal DNA flanking the miniTn*5*(*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,

Table 2. Primers

Name	Position	Sequence (5′ to 3′)
hth1	+697 to +715	CGCGCCGCCTGGATTGTCA
hth2	+1020 to +1002	CCGGCCGGCGATGGACTTG
hth3	+1545 to +1568	TACCAGGGAGGCGCTACGGACGAA
hth4	+1948 to +1926	AGGCTGGGGCGCTGGAAGAAGAT
hth5	+731 to +750	TGGTGCACGCCAACAATGCC
hth6	+1250 to +1230	CTGCGGGTCCCCAATGACGAG
hth7	+978 to +1000	GCACATCATCCAGGGCGAGACGA
hth8	+2668 to +2646	AACCGTCGACCCAGGCGCGGACGGTGTTCG
hthF	+697 to +715	CGCGCCGCCTGGATTGTCA
hthR	+761 to +741	CGCGCTCGGCCGGCATTGTTG

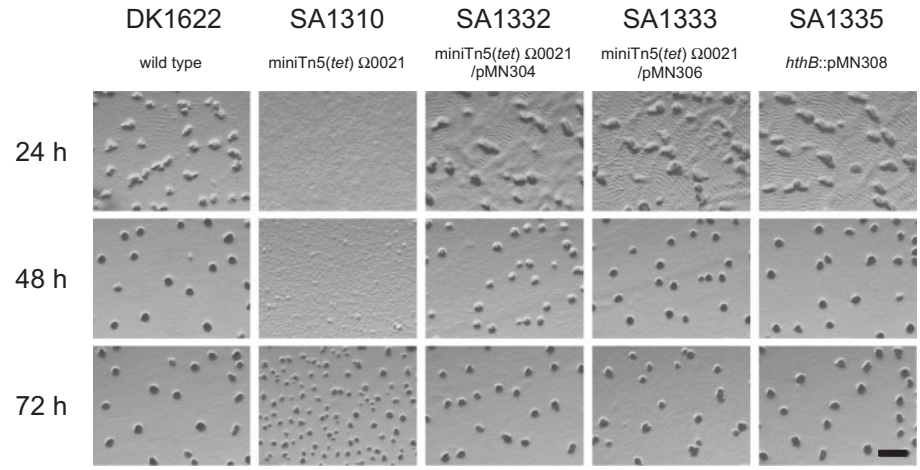

Fig. 1. Developmental phenotypes of *hthAhthB* and *hthB* mutants. The indicated strains were exposed to starvation on CF agar for the indicated periods of time and viewed in a Leica MZ8 stereomicroscope. The genotypes of the strains are indicated below their names. SA1310 is *hthAhthB*, SA1332 is *hthA*$^+$*hthB*, SA1333 is *hthA*$^+$*hthB*$^+$, and SA1335 is *hthA*$^+$*hthB*. Bar, 0·2 mm.

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-Ω0021, -Ω2525, -Ω4074, -Ω4409 and -Ω4695) 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*) Ω0021, 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*) Ω0021, Ω2525, Ω4074, Ω4409 and Ω4695 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*) Ω0021, 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

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 %.

Strain	Genotype	Sporulation frequency at:	
		72 h	120 h
DK1622	Wild-type	30 ± 8	100 ± 12
SA1310	*hthA*::miniTn*5*(*tet*) Ω0021	0·08 ± 0·03	20 ± 3
SA1332	*hthA*::miniTn*5*(*tet*) Ω0021/pMN304	0·10 ± 0·03	19 ± 3
SA1333	*hthA*::miniTn*5*(*tet*) Ω0021/pMN306	38 ± 10	85 ± 16
SA1335	*hthB*::pMN308	0·05 ± 0·03	18 ± 3

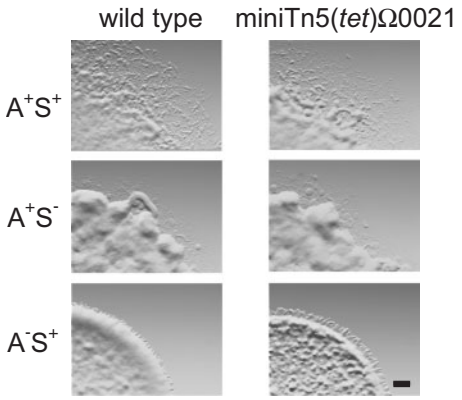

Fig. 2. Effect of an *hthAhthB* mutation on vegetative motility. Cells of the various strains were spotted on 1·5% agar supplemented with 0·5% CTT and viewed in a Leica MZ8 stereomicroscope. Swarm edges were recorded after 24 h of incubation at 32 °C. Strains in the left vertical column are all *hthA*$^+$*hthB*$^+$; strains in the right vertical column carry miniTn*5*(*tet*) Ω0021 and are all *hthAhthB*. 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*::miniTn*5*(*tet*) Ω0021), SA1312(*sglG1*, *hthA*::miniTn*5*(*tet*) Ω0021) and SA1311 (*aglB1*, *hthA*::miniTn*5*(*tet*) Ω0021). Bar, 0·5 mm.

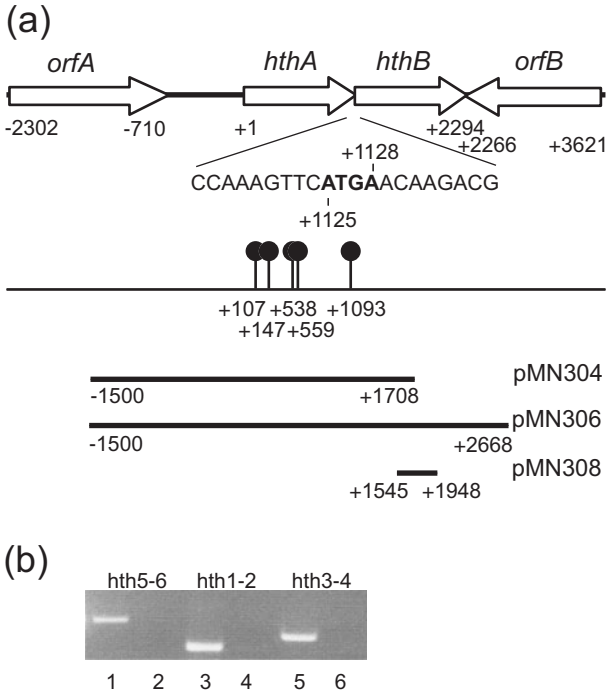

Fig. 3. Analysis of the *hthAB* region. (a) Open reading frames are indicated by open arrows. Arrows indicate the direction of transcription. Coordinates are relative to +1, the first nucleotide in the start codon of *hthA*. Below the physical map, the overlap between the *hthA* stop codon and *hthB* start codon is shown. The lollipops indicate the sites of insertion of miniTn5(*tet*) in *hthA*. miniTn*5*(*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 (miniTn*5*(*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).

is similar to that of the five original mutants, these data suggest that the miniTn*5*(*tet*) insertions are responsible for the mutant phenotype. From here on, only data for the mutant strain, which carries miniTn*5*(*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 miniTn*5*(*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 miniTn*5*(*tet*) Ω0021 insertion did not generate a non-swarming phenotype when crossed into A$^-$ or S$^-$ mutant backgrounds (Fig. 2). Thus, the miniTn*5*(*tet*) Ω0021 insertion does not interfere with either the A- or the S-motility system in vegetative cells, suggesting that the gene carrying the miniTn*5*(*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 miniTn*5*(*tet*) Ω0021 insertion are not secondary to an effect on motility.

Analysis of the *hth* locus

Analyses of the DNA sequences surrounding the insertion sites of miniTn*5*(*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). miniTn*5*(*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 FixJ 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 FixJ-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 miniTn*5*(*tet*) into a gene is likely to inactivate it. Moreover, miniTn*5*(*tet*) may have polar effects on downstream genes. Thus, in the case of miniTn*5*(*tet*) Ω0021, the insertion may inactivate *hthA* and have a polar effect on *hthB*. To determine whether miniTn*5*(*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, miniTn*5*(*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.

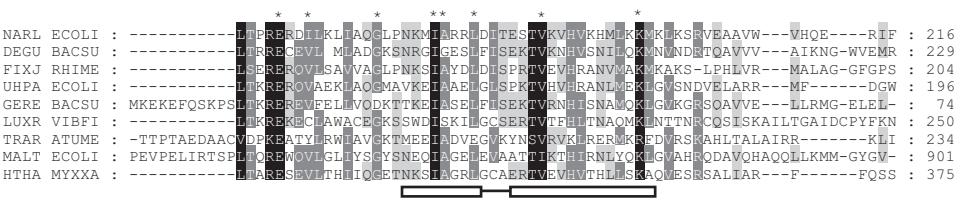

Fig. 4. Alignment of the C-terminal part of HthA with the DNA-binding domains in NarL from *E. coli* (CAA48935), DegU from *B. subtilis* (RGBSXD), FixJ from *R. meliloti* (P10958), UhpA from *E. coli* (P10940), GerE from *B. subtilis* (P11470), LuxR from *V. fischeri* (P12746), TraR from *A. tumefaciens* (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 miniTn*5*(*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 *hthA* (Fig. 3a). pMN304 and pMN306 were introduced by electroporation into SA1310, which carries the miniTn*5*(*tet*) Ω0021 insertion, to give strains SA1332 and SA1333, respectively. In both strains, the plasmid had integrated by homologous recombination upstream from the miniTn*5*(*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 DK1622 and SA1310. As shown in Fig. 1 and Table 3, the aggregation defect caused by the miniTn*5*(*tet*) Ω0021 insertion was corrected by pMN304 in SA1332. However, pMN304 did not correct the sporulation defect caused by miniTn*5*(*tet*) Ω0021. On the other hand, both the aggregation defect and the sporulation defect caused by miniTn*5*(*tet*) Ω0021 were corrected by pMN306 in SA1333. These observations show that the developmental defects caused by miniTn*5*(*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 pBGS18 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*::pMN308 mutation, SA1335 was exposed to starvation on CF starvation medium in parallel with the wild-type strain DK1622 and SA1310, which carries miniTn*5*(*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 miniTn*5*(*tet*) Ω0021 was cell-autonomous, cells of SA1310 were co-developed with an equal number of wild-type cells

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.

Test strain (genotype)	Sporulation frequency of test strain during co-development with:		
	No other strain	DK1622 (wild-type)	SA1310 (*hthA*::miniTn*5*(*tet*) Ω0021)
SA1310 (*hthA*::miniTn*5*(*tet*) Ω0021)	0·08 ± 0·03	0·07 ± 0·03	NA
DK4398 (*asgB*, Tn*5lac* Ω4411)	5·5 ± 1·2	33 ± 3	100 ± 17
DK11015 (*bsgA*::Tn*5 lac* Ω7516)	< 0·0001	0·004 ± 0·003	0·003 ± 0·001
LS269 (*csgA*::Tn*5lac* Ω269)	< 0·0001	0·23 ± 0·15	0·31 ± 0·07
DK3260 (*dsg429* Tn*5* Ω1867)	< 0·0001	40 ± 5	40 ± 13
JD300 (*esg*::Tn*5* Ω258)	< 0·0001	0·20 ± 0·11	0·27 ± 0·15

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 miniTn*5*(*tet*) Ω0021 are cell-autonomous and that miniTn*5*(*tet*) Ω0021 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 miniTn*5*(*tet*) Ω0021 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 miniTn*5*(*tet*) Ω0021 insertion on developmental gene expression, the mutation was transduced into the eight strains, each containing a *lacZ* reporter fusion. Subsequently, isogenic *hthA*$^+$ *hthB*$^+$ 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 Tn*5lac* Ω4521, *csgA–lacZ* (pJM200), Tn*5lac* Ω4499, Tn*5lac* Ω4403 and Tn*5lac* Ω4401 were indistinguishable in the two strains. The three fusions Tn*5lac* Ω4408, Tn*5lac* Ω7540 and Tn*5lac* Ω4414 had altered expression profiles in the *hthAhthB* strain. In the case of Tn*5lac* Ω4408, 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 Tn*5lac* Ω7540, 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 Tn*5lac* Ω4414, 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.

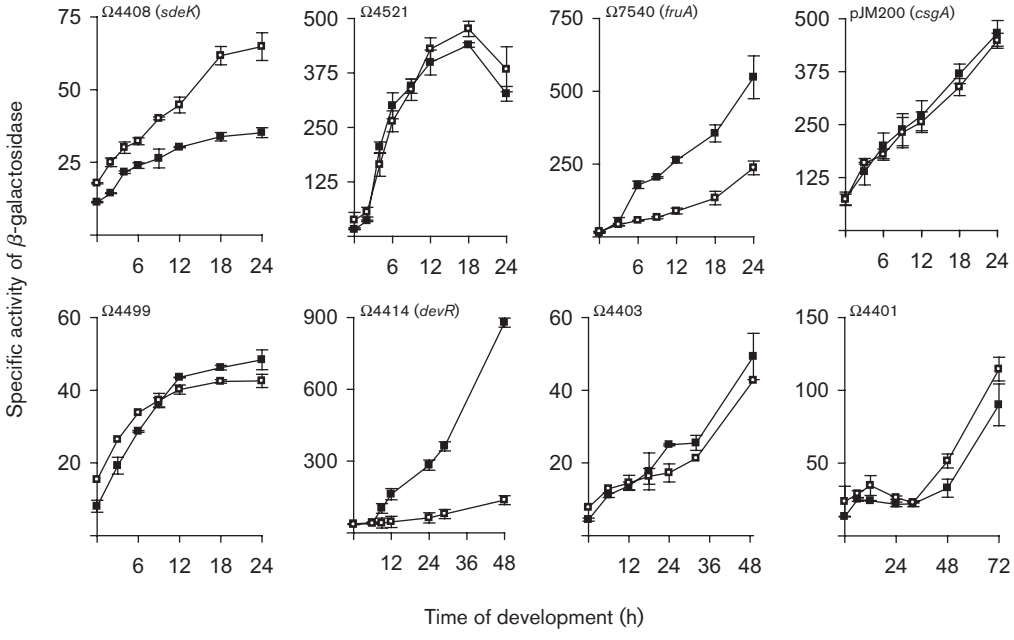

Fig. 5. Effect of *hthAhthB* mutation on developmental gene expression. Expression of the indicated *lacZ* fusions in wild-type DK1622 cells (■) and in *hthAhthB* SA1310 cells (□). Cells were starved on CF agar, samples withdrawn at the indicated time points and specific activities of β-galactosidase determined. The experiments were done in triplicate. Error bars indicate standard deviations. Specific activities of β-galactosidase are given in nmol *o*-nitrophenol min^{-1} (mg protein)$^{-1}$.

DISCUSSION

Aggregation and sporulation are the two major morphogenetic 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 the proteins (Parkinson & Kofoid, 1992); binding of the cognate AHL autoinducer to the N-terminal domain modulates the transcriptional activity of the LuxR-like transcriptional regulators (Fuqua *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 (Tn*5lac* 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 (Tn*5lac* Ω4408) in non-starving cells and during development. SdeK encodes a histidine protein kinase important for fruiting body formation (Garza *et al.*, 1998; Pollack & 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* (Tn*5lac* Ω7540) and *devR–lacZ* fusions (Tn*5lac* 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 (Tn*5lac* Ω4414) (Ellehauge *et al.*, 1998; Kroos *et al.*, 1990) and Tn*5lac* Ω4403 (E. Ellehauge, unpublished; Pollack & Singer, 2001). Therefore, the expression profile of the *devR–lacZ* fusion (Tn*5lac* 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 Tn*5lac* 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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