J. Microbiol. Biotechnol. (2003), **13**(3), 400–405

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

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HpkA, a Histidine Protein Kinase Homolog, is Required for Fruiting Body Development in *Myxococcus xanthus*

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Received: November 8, 2002
Accepted: March 30, 2003

Abstract A gene (*hpkA*), encoding a histidine protein kinase homolog, has been identified in the upstream region of the *espAB* operon in *Myxococcus xanthus*. It encodes a 333 amino acid (35,952 Da) protein with a histidine protein kinase domain in the region from amino acid 90 to 317. Null mutations in the *hpkA* gene caused formation of loose irregular fruiting bodies, while wild-type strains developed tight hemispherical fruiting bodies under developmental conditions. Sporulation of the *hpkA* mutant was delayed by at least 12 h compared to that of the wild-type. It appeared that the *hpkA* mutation increased the expression of the *espAB* operon by more than 2-fold compared with the wild-type under developmental conditions. Expression of the *hpkA* gene was low under vegetative conditions, but was highly induced under developmental conditions.

Key words: Myxobacteria, *Myxococcus xanthus*, *hpkA*, *espA*, fruiting body, sporulation

A Gram-negative soil bacterium, *Myxococcus xanthus*, is well known to develop multicellular fruiting bodies under starved conditions [6, 19, 23]. Fruiting body development consists of two major morphogenic processes: one for aggregation of a group of cells to form a raised mound (multicellular morphogenesis) and the other for sporulation of individual cells into spherical myxospores (cellular morphogenesis) [4, 14, 24]. During early fruiting body development, a group of more than 10^5 starved cells aggregate to form a raised translucent mound of cells [8]. Individual rod-shaped cells then transform into environmentally resistant spherical spores in the mound, resulting in a mature fruiting body [24]. Since sporulation of individual cells requires the raised mound, cells normally do not sporulate outside of the mound but sporulate only in the mound. The spores in the fruiting body germinate simultaneously if nutrients are available, resulting in the formation of a swarm of vegetative cells again [7]. Although the aggregation and sporulation pathways are separated, they appear to be coordinated to form mature fruiting bodies [4, 12]. A histidine protein kinase, EspA, and a membrane protein, EspB, are known to be a part of a mechanism that monitors aggregation status and regulates initiation of sporulation [4]. Null mutations in the *espA* gene cause aggregation independent sporulation, and null mutations in the *espB* gene cause delayed sporulation with low sporulation efficiency [4]. The *espA* and *espB* genes compose an operon, which is expressed only under developmental conditions. In this paper, the identification of a gene, *hpkA*, encoding a putative histidine protein kinase in a DNA region upstream of the *espAB* operon is reported. The *hpkA* gene was developmentally regulated and required for fruiting body formation. It appears that the HpkA protein played a role in the expression of the *espAB* operon.

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MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Culture Conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *M. xanthus* was cultured vegetatively in CYE medium, which contains 1% (w/v) casitone, 0.5% yeast extract, 10 mM morpholinepropanesulphonic acid (MOPS, pH 7.6), and 8 mM $MgSO_4$ [22]. Fruiting body development of *M. xanthus* was initiated by placing a 20 µl spot of 5×10^9 cells/ml on a CF plate containing 1.5% agar. CF medium contains 10 mM MOPS (pH 7.6), 0.015% casitone, 8 mM $MgSO_4$, 1 mM KH_2PO_4, 0.2% sodium citrate, and 0.1% pyruvate [22]. Liquid cultures were incubated at 32°C with shaking at 275 rpm. Solid culture plates were incubated at 34°C.

Table 1. Strains and plasmids used in this study.

Strains and plasmids	Relevant feature	Source or references
M. xanthus		
DZ2	Wild-type	2
DZ4224	DZ2::pKY564, *espA-lacZ564*	4
DZ4233	DZ2::pKY565, *asgD-lacZ565*	3
DZ4284	DZ2::pKY598, *pknD1-lacZ598*	Lab collection
KYC292	DZ2::pKY569, *hpkA569*	This study
KYC352	DZ2::pKY600, *hpkA-lacZ600*	This study
KYC363	DZ2, Δ*hpkA605*	This study
KYC379	KYC363::pKY564, Δ*hpkA605 espA-lacZ564*	This study
KYC380	KYC363::pKY598, Δ*hpkA605 pknD1-lacZ598*	This study
KYC448	KYC363::pKY565, Δ*hpkA605 asgD-lacZ565*	This study
Plasmids		
pKY334	Derivative of pZErO-2 carrying 9 kb DNA from DZ4213	4
pKY480	Cloning vector, $SacB^{+}$, Km^{R}	3
pKY481	Used to create a *lacZ* translational fusion, Km^{R}	3
pKY564	Derivative of pKY481 carrying the *espA-lacZ564* translational fusion	4
pKY565	Derivative of pKY481 carrying the *asgD-lacZ565* translational fusion	3
pKY569	Derivative of pKY481 carrying an internal PCR fragment of *hpkA*	This study
pKY598	Derivative of pKY481 carrying the *pknD1-lacZ598* translational fusion	Lab collection
pKY600	Derivative of pKY481 carrying the *hpkA-lacZ600* translational fusion	This study
pKY605	Derivative of pKY480 used to create Δ*hpkA605*	This study

DNA Manipulations and Sequence Analysis

DNA was manipulated using standard protocols [20]. Oligonucleotides were synthesized at Bioneer Co. Basic Local Alignment Search Tool (BLAST) was used for homology searches [1]. Simple Modular Architecture Research Tool (SMART) was used to analyze functional domains of proteins [21]. GeneDoc was used for multiple sequence alignment [15]. The *hpkA* nucleotide sequence has been deposited in the GenBank DNA sequence database (Accession number, AF163841).

Plasmid Construction

pKY569 is a derivative of pKY481, carrying an internal PCR fragment of *hpkA*, which was amplified using two oligonucleotides, 5'-CAGGATCCGCGGCAGGCCCTC-ACACTCCAC-3' and 5'-GCACTCGAGGCGCCGCGG-TCCTCCACACG-3', as primers and pKY334 as a DNA template. pKY600 is a derivative of pKY481, carrying a translational fusion between the 17th codon of *hpkA* and the 8th codon of *lacZ*. The fusion was first created *in vitro* by cloning a PCR fragment, containing a region between 478 bp upstream and 50 bp downstream from the putative *hpkA* translational start site into the *Xho*I and *Bam*HI sites of pKY481. The PCR fragment was amplified using two oligonucleotides, 5'-GACCTCGAGCTCGAACCCCGC-CCACTCGGA-3' and 5'-GACGGATCCCGCCGCTGTG-CCACCGCCGA-3', as primers and pKY334 as the DNA template. pKY605, which was used to create an in-frame deletion of *hpkA*, was constructed by the reported method [13]. Four oligonucleotides were used to amplify a PCR fragment carrying an *hpkA* in frame deletion: 5'-GACC-TCGAGCTCGAACCCCGCCCACTCGGA-3' (N1), 5'-ACGGTCGCAGTACGATCCTGTGCGCCGCTGTGCC-ACCGCCGA-3' (N2), 5'-ACAGGATCGTACTGCGAC-CGTTTGCCCGTGGCCGGCGCCTTC-3' (C1), and 5'-GACGGATCCTCGGCTGGCCATTGCCGGAAC-3' (C2). Primers N1 and N2 were used to amplify an N-terminal PCR fragment, and primers C1 and C2 were used to amplify the C-terminal PCR fragment. The resultant two PCR products and primers N1 and C2 were then used to amplify a final PCR product, carrying an *hpkA* in-frame deletion. The final PCR fragment was then ligated into pKY480, after it was digested with *Bam*HI and *Xho*I, to generate pKY605.

Creation and Characterization of Mutations in *M. xanthus*

Plasmid insertion and in-frame deletion mutations were performed with the methods described previously [3, 5]. Fruiting bodies were observed with a Nikon SMZ1000 stereomicroscope and individual cells and spores were observed with a Leica DMLB microscope. Microscopic images were captured with a Nikon Coolpix-995 digital camera. Sonication-resistant spores were counted using a hemacytometer, after vegetative cells were disrupted by sonication. β-Galactosidase activity was assayed with a protocol that has been described previously [9, 11].

RESULTS AND DISCUSSION

Identification of *hpkA*, Which is Required for Normal Fruiting Body Formation

The *espAB* operon encodes histidine protein kinase, EspA, and a putative membrane bound protein, EspB. The EspA and EspB proteins play an important role in controlling sporulation initiation timing during fruiting body formation of *M. xanthus* [4]. In the course of characterizing the region upstream of the *espAB* operon, an open reading frame (ORF) was identified whose deduced protein product was very similar to the EspA protein, a histidine protein kinase. Since the deduced protein product was a histidine protein kinase homolog, the ORF was designated as *hpkA*.

Since the *hpkA* gene located near the *espAB* operon showed high similarity to the EspA protein, it was possible that the product of *hpkA* might have a functional relation with the EspAB system. To test this possibility, insertion and in-frame deletion mutants of the *hpkA* gene were created and compared to the developmental phenotype of the mutant with the wild-type strain of *M. xanthus*, DZ2. The insertion mutant, KYC292, was created by a plasmid insertion in the middle of the *hpkA* gene and the in-frame deletion mutant, KYC363, was created by deleting 89% of the gene (amino acids 18-312) from the chromosome. When the cells were placed under developmental conditions, the wild-type strain DZ2 formed tight hemispherical fruiting bodies (Fig. 2). However, the mutants KYC292 and KYC363, formed loose, irregular fruiting bodies under the same conditions (Fig. 2). Sporulation of the mutants was also defected. Under the conditions where the wild-type strain, DZ2, produced 4.9×10^7 spores, the *hpkA* in-frame deletion mutant, KYC363, produced 1.4×10^7 spores, 29% of the wild-type level. It also appears that the sporulation of KYC363 was delayed at least 12 h compared to that of the wild-type. DZ2 started to produce sonication-resistant refractile spores in 36 h, but KYC363 did not produce any spores until 48 h. Therefore, these results suggest that the *hpkA* gene is required for normal fruiting body development in *M. xanthus*. Meanwhile, an insertion mutation in an ORF, *orfD*, located just upstream of the *hpkA* gene did not cause any developmental defects, suggesting that the expression of *hpkA* should be independent from *orfD* (Fig. 1).

Similarity of HpkA to Other Proteins

The deduced *hpkA* gene product, HpkA, contained 333 amino acids. Sequence analysis using SMART revealed two functional domains, a histidine kinase-A domain (HisKA) in a region from amino acid 90 to amino acid 164, and a histidine kinase-like ATPase (HATPase_c) domain in a region from amino acid 204 to amino acid 317 (Fig. 3). It has been known that the HisKA and HATPase_c domains constitute a histidine protein kinase in many proteins involved in bacterial signal transduction [16]. The region from amino acid 79 to amino acid 316 of the HpkA protein, which contained the histidine protein kinase domain, was 51%, 53%, 33%, and 26% identical to the histidine protein kinase domain of EspA [4], MokA [10], and AsgA [18] from *M. xanthus*, and KinA from *Bacillus subtilis* [17], respectively (Fig. 3). EspA has been known to be required for regulation of sporulation timing during fruiting body development, and MokA has been reported to be required for fruiting body development and osmotic tolerance

Fig. 1. A physical map of the region containing the *hpkA*, *orfD*, *pknD1*, *espA*, and *espB* genes.

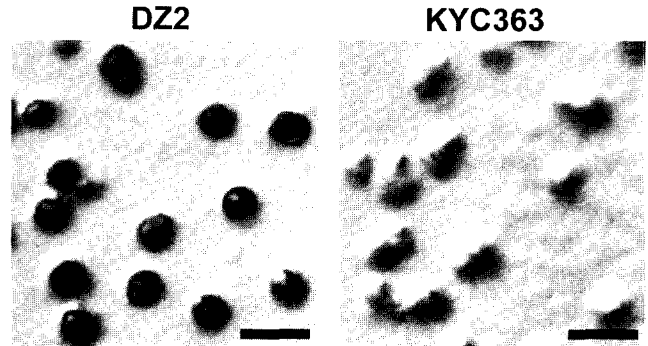


Fig. 2. Effects of the *hpkA* null mutations on fruiting body development.

Twenty µl spots (5×10^9 cells/ml) of DZ2 (wild-type) and KYC363 ($\Delta hpkA$) were placed on CF plates and incubated at 34°C for 3 days. Bars, 0.2 mm.

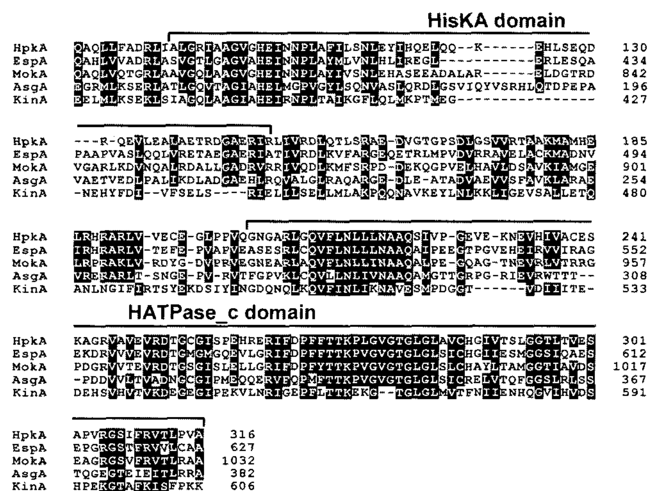


Fig. 3. Domain organization and sequence alignment of HpkA with other homologous proteins.

The histidine protein kinase domain of HpkA, which consists of a histidine kinase-A domain (HisKA) and a histidine kinase-like ATPase (HATPase_c), was aligned with the histidine protein kinase domains of EspA [4], MokA [10], and AsgA [18] from *M. xanthus*, and KinA [17] from *Bacillus subtilis*.

[4, 10]. Although HpkA was most similar to MokA, the *hpkA* null mutant did not show any growth defect under osmotically stressful conditions where the growth of the *mokA* mutant was inhibited (data not shown).

Expression of *hpkA*

Expressions of the genes required for fruiting body development are generally induced under developmental conditions [11]. Since the *hpkA* gene appears to be required for normal fruiting body formation, how the expression of *hpkA* was regulated was studied using a strain carrying an *hpkA-lacZ* translational fusion. The fusion was first created *in vitro*, and then introduced into the chromosome of *M. xanthus* in a way that did not disrupt *hpkA*, as described in Materials and Methods. Therefore, the vegetative and developmental phenotype of the resultant strain, KYC352, was indistinguishable from the wild-type strain, DZ2, except it produced β-galactosidase from the fusion (data not shown). Under vegetative conditions, KYC352 carrying the *hpkA-lacZ* fusion showed a low level of β-galactosidase activity. However, when the cells of KYC352 were placed under developmental conditions, the level of β-galactosidase activity continuously increased until 48 h as shown in Fig. 4. Therefore, these results suggest that the expression of *hpkA* is developmentally regulated, although it is still expressed at a low level under vegetative conditions.

Effect of the *hpkA* Null Mutation on Expression of the *espAB* Operon

The *espAB* operon is developmentally regulated and is not normally expressed during vegetative growth [4]. In the stationary phase of the vegetative growth, however, the *espAB* operon is expressed at a very low level. To elucidate the possible functional relationship between *hpkA* and

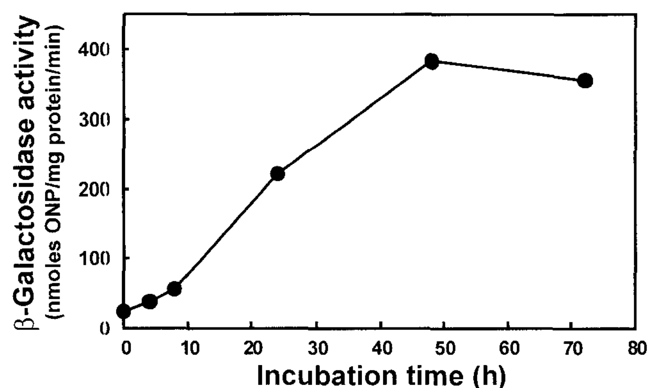
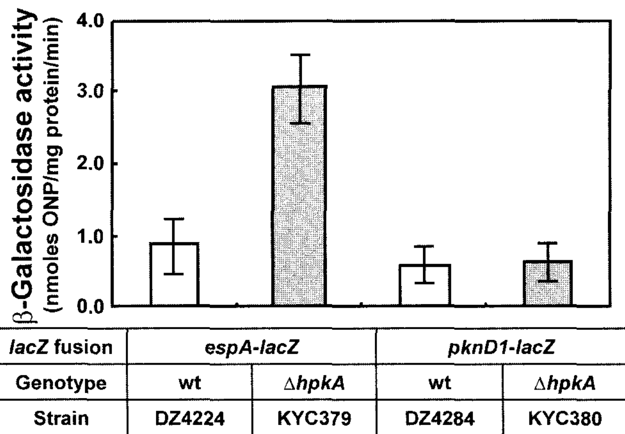


Fig. 4. Expression of the *hpkA-lacZ* fusion under developmental condition.

Twenty μl spots (5×10^9 cells/ml) of KYC352 carrying the *hpkA-lacZ* fusion was placed on CF plates and incubated at 34°C. The cells were harvested at different time points and β-galactosidase activity was measured as previously reported [11].



lacZ fusion	*espA-lacZ*		*pknD1-lacZ*	
Genotype	wt	Δ*hpkA*	wt	Δ*hpkA*
Strain	DZ4224	KYC379	DZ4284	KYC380

Fig. 5. Effect of the Δ*hpkA* mutation on the expression of the *espA-lacZ* and *pknD1-lacZ* fusions.

Twenty μl spots of cell suspension (5×10^9 cells/ml) were placed on CYE plates and incubated at 34°C. Cells were scraped after 48 h and β-galactosidase activity was measured. Experiments were repeated four times and the average activities are shown.

espA, effects of the mutation of one gene on the expression of other gene and vice versa were tested. When the *hpkA-lacZ* fusion was introduced into the Δ*espA* mutant, the expression of β-galactosidase specific activity was not changed at all (data not shown). However, when the *espA-lacZ* fusion was introduced into the Δ*hpk*A mutant, it was observed that the level of β-galactosidase activity was increased (Fig. 5). β-Galactosidase activity of DZ4224, which carried the *espA-lacZ* fusion in the wild-type background, was 0.9 nmol ONP/mg protein/min after 48 h of incubation on a CYE plate. However, under the same conditions, β-galactosidase activity of KYC379, which carried the *espA-lacZ* fusion in the Δ*hpkA* mutant background, increased to 3.1 nmol ONP/mg protein/min, a 3.4-fold increase compared with DZ4224. Since it was possible that the effect of the Δ*hpkA* mutation was pleotropic rather than specific to the *espA-lacZ* fusion, the effect of the Δ*hpkA* mutation on the expression of another fusion, *pknD1-lacZ* fusion, which was also developmentally regulated, was tested. However, the expression of the *pknD1-lacZ* fusion was not affected by the Δ*hpkA* mutation under the same conditions (Fig. 5). In addition, *asgD*, which is another developmentally regulated gene [3], was also not affected by the Δ*hpkA* mutation under the same conditions (data not shown). This, therefore, suggested that the effect on the expression of the *espA-lacZ* fusion by the Δ*hpkA* mutation might be pathway-specific. However, it is not clear at this point whether the effect of the Δ*hpk*A mutation on the expression of the *espA-lacZ* fusion is direct or indirect.

When KYC379 carrying the *espA-lacZ* fusion in the Δ*hpkA* mutant background was placed under developmental conditions, expression of the fusion was induced as if it were in the wild-type background (Fig. 6). However, the

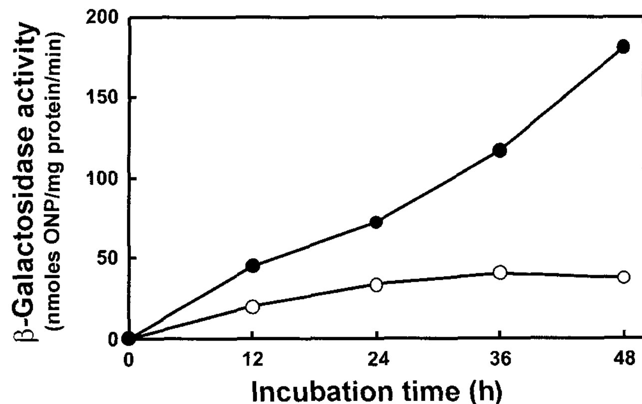


Fig. 6. Effect of the Δ*hpkA* mutation on the expression of the *espA-lacZ* fusion under developmental condition.
Twenty μl spots (5×10^9 cells/ml) of DZ4224 (○; wild-type) and KYC379 (●; Δ*hpkA*) carrying the *espA-lacZ* fusion were placed on CF plates and incubated at 34°C. The cells were harvested at different time points and β-galactosidase activity was measured.

expression of the β-galactosidase activity in the *hpkA* mutant was 2~3-fold higher than that of the wild-type (Fig. 6). This indicated that the HpkA protein had inhibitory effects on the expression of the *espAB* operon under the developmental condition. However, the β-galactosidase activity was induced in the *hpkA* mutant, showing that the inhibitory activity was not 100%. This might suggest that the HpkA protein is not a major regulatory protein for the expression of the *espA*B operon during fruiting body development. However, it is also possible that HpkA requires a signal for full inhibitory activity, such as nutrients, as observed in many other bacterial signal transduction systems. Fruiting body development of *M. xanthus* is aborted if environmental conditions are favorable again. Since the expression of *hpkA* is developmentally regulated, it is possible that the HpkA protein is part of a mechanism that aborts the expression of the *espAB* operon-related genes in response to the environmental conditions during fruiting body development. The *hpkA* mutant showed delayed sporulation with low sporulation efficiency. It is tempting to speculate that the *hpkA* mutant phenotype might elevate an expression of the EspA protein, which has been known to inhibit sporulation initiation. Delayed sporulation with low sporulation efficiency is also the characteristic phenotype of the *espB* mutant [4].

In summary, a gene, *hpkA*, encoding a putative histidine protein kinase has been identified. Genetic studies have indicated that the HpkA protein plays a role in the expression of the *espAB* operon. Since the EspA and EspB proteins play an important role in sporulation initiation, characterization of the HpkA protein will be helpful to understand the sporulation process of individual cells, one of the major events occurring during the fruiting body development of *M. xanthus*.

Acknowledgment

This work was supported by Korea Research Foundation Grant (KRF-2001-015-DP0435).

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