Transcriptional Activation of a Heat-shock Gene, \( \text{lonD} \), of \textit{Myxococcus xanthus} by a Two Component Histidine-Aspartate Phosphorelay System*

Received for publication, October 22, 2001, and in revised form, December 14, 2001
Published, JBC Papers in Press, December 17, 2001, DOI 10.1074/jbc.M110155200

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In vitro transcription of \( \text{lonD} \), a heat-shock gene from \textit{Myxococcus xanthus}, was stimulated in the presence of extract from heat-shocked cells. For this stimulation the upstream promoter region of \( \text{lonD} \) was found to be essential. Activation of \( \text{lonD} \) transcription was also observed when extract from non-heat-shocked cells was heat treated in vitro at 42 °C for 10 min. A DNA binding assay and footprinting analysis revealed that a factor(s) binds to the upstream region from -122 to -107 with respect to the transcription initiation site. This region was required for heat-shock induction of \( \text{lonD} \) expression both in vitro and in vivo. The \( \text{lonD} \) promoter-binding protein named HsfA was purified, and its gene was cloned. Analysis of the DNA sequence reveals that HsfA is a response regulator of the two-component system and shows high sequence similarity to the NtrC family or the enhancer-binding proteins. Upstream of hsfA, a gene encoding a histidine kinase was identified and named hsfB. HsfB was found to be autophosphorylated and able to phosphorylate HsfA. HsfA with HsfB activated in vitro transcription of \( \text{lonD} \) in a manner dependent on RNA polymerase containing SigA, the housekeeping sigma factor of \textit{M. xanthus}.

The major pathway of signal transduction required for response and adaptation to environmental changes in prokaryotes consists of the two-component His-Asp phosphorelay system (1). This system basically utilizes two protein components, a sensor histidine kinase and a response regulator. Sensors typically contain a C-terminal transmitter module or a histidine kinase domain coupled to an N-terminal input domain. Response regulators typically contain an N-terminal receiver domain coupled to a C-terminal output domain. The mechanisms of transmitter-receiver communication involve phosphorylation and dephosphorylation reactions. Transmitters or histidine kinases have an autokinase activity that phosphorylates a specific histidine residue in the presence of ATP. The product phosphohistidine serves as a high energy intermediate for subsequent transfer of the phosphate group to a specific aspartate residue in the receiver domain. Response regulators become active upon receiving the phosphate group and generally function as transcription factors for cognate gene expression.

Among various stress responses, the heat-shock response is the most extensively studied. Transcription of heat-shock genes is regulated in different fashions in bacteria (2–5). In \textit{Escherichia coli}, RNA polymerase (RNAP)\(^1\) containing an alternative sigma factor, RpoH (\(\sigma^H\), \(\sigma^S\)) recognizes promoters of most of heat-shock genes and initiates their transcription (2, 5). In addition to RpoH, RNAP containing RpoE (\(\sigma^E\)) transcribes other heat-shock genes that are induced at higher temperature (50 °C) (2, 5). \textit{Bacillus subtilis} utilizes rather complex mechanisms for heat-shock response transcription. Heat-shock genes are classified into four groups (Class I–IV) (3, 4). Class I and III genes are negatively regulated by the CIRCE element and HrcA, and in tandem repeated DNA sequences and CtsR, respectively. Class II genes are transcribed by RNAP containing an alternative sigma factor, SigB. The mechanism for transcription of Class IV genes is not known. Both RpoH-driven transcription and negative regulation by CIRCE and HrcA are found in \textit{Bradyrhizobium japonicum} (4). Furthermore, another type of negative regulation controls some heat-shock genes by ROSE, a DNA element of approximately 100 bp in length and a putative repressor (4).

\textit{Myxococcus xanthus} is a Gram-negative bacterium that can differentiate through fruiting body formation into spores upon starvation (6, 7). It was found that \textit{M. xanthus} contains at least eight sigma factor genes, sigA (8), sigB (9), sigC (10), sigD (11), sigE (12), rpoEI (13), rpoV (14), and carQ (15). SigB, SigC, and SigE show sequence similarity to RpoH. However, they are not induced by heat-shock, and even the triple deletion of these genes does not affect production of heat-shock proteins by heat-shock (12).

To understand heat-shock response transcription in \textit{M. xanthus}, we first identified the \( \text{lonD} \) gene as a heat-shock gene. The \( \text{lonD} \) gene has been shown to be essential for \textit{M. xanthus} fruiting body development (16, 17). Using the \( \text{lonD} \) gene, we have purified a DNA-binding protein specific to the \( \text{lonD} \) promoter. This DNA-binding protein belongs to the NtrC family or enhancer-binding proteins. Subsequent analysis revealed that a histidine kinase is also involved in \( \text{lonD} \) expression.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Growth Conditions**

\textit{M. xanthus} DZF1 was grown in CYE liquid medium (18). \textit{E. coli} JM83 (19) was used as a recipient strain for transformation and grown at 37 °C in LB medium (20) supplemented with 50 µg/ml proper antibiotics when necessary. \textit{E. coli} BL21(DE3) (21) was used as a recipient

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*This work was supported by a grant from the Foundation of University of Medicine and Dentistry of New Jersey. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: RNAP, RNA polymerase; AS, ammonium sulfate; H-AS, AS fraction prepared from heat-shocked cells; NH-AS, AS fraction prepared from non-heat-shocked cells; RNAP/SigA, RNAP containing SigA; ORF, open reading frame.

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Printed in U.S.A.
strain for transformation to overexpress genes cloned in pET24b and grown at 37 °C in LB medium supplemented with 50 μg/ml kanamycin.

Preparation of AS Fractions

*M. xanthus* DZ1F1 cells were grown in CYE liquid medium at 30 °C. Exponentially growing cells (Klett units of 80) at 30 °C were heat-shocked at 42 °C for 10 min. Cell extracts were prepared as described by Gross et al. (22). The step (iv) extracts were diluted by 5-fold and precipitated by ammonium sulfate (40–65%). The precipitates were suspended in the buffer containing 10 mM Tris-HCl (pH 8.0), 10% glycerol, 0.1 mM EDTA, 0.1 mM diithiothreitol, protease inhibitors (Roche), and 0.1 M KCl. This extract is referred to as AS fraction.

In Vitro Transcription

Two different templates contain the lonD promoter region from the −346 base to the +96 base (plonD346) and from −41 to +96 (plonD41) for in vitro transcription analysis. The in vitro transcription reaction was carried out in buffer (50 mM Tris-HCl (pH 8.0), 10 mM MgCl2, 50 mM KCl, 0.1 mM EDTA, 0.1 mM diithiothreitol, 25 μM [γ-32P]ATP, 0.5 unit/μl RNase inhibitor (Roche)) containing 0.5 mg/ml plasmid DNA templates in a 20-μl total volume. The reaction was first incubated at 30 °C for 10 min. Then 2 μl of AS fraction was added, and incubation was continued for an additional 15 min. When purified proteins were used, HsfB was first incubated in the buffer supplemented with 1 μM ATP for 2 min at 30 °C, then HsfA was added and incubated for 5 min at 30 °C. RNAP containing SigA was added, and incubation was continued for another 15 min at 30 °C. The reaction was stopped by the addition of 40 μl of stop solution (1 M ammonium acetate, 40 mM EDTA, 0.4 mg/ml tRNA). The mixtures were extracted with phenol/chloroform/isomyl alcohol, and transcripts were precipitated with ethanol. Transcripts were resuspended in distilled water and analyzed by primer extension analysis as described previously (11).

DNA Binding Assay and Footprinting Analysis

Plasmid plonD346–42 containing the lonD promoter region from the −346 base to the −42 base was prepared for the DNA binding assay. The DNA fragments were prepared by digesting plonD346–42 with proper enzymes and purified with 5% polyacrylamide gel. The enzymes used for each experiment are described in the text or figure legends. The DNA fragments were labeled with [ω-32P]dCTP by Klenow fragment.

DNA binding assays were performed in 10 μl of the reaction mixture containing 10 mM Tris-HCl (pH 8.0), 50 mM KCl, 1 mM dithiothreitol, 10 μg/ml bovine serum albumin, 10% glycerol, 1 μg of double-stranded poly(dI-dC) (Amersham Biosciences, Inc.), 1 ng of *pho* labeled DNA fragments, and 50 μg of extracts. The mixture was incubated at 30 °C for 10 min and loaded onto a 5% polyacrylamide; binding patterns were analyzed by autoradiography. Footprinting was carried out by using 1,10-phenanthroline-copper as described by Ruwabara and Sigman (23).

Purification of the lonD Promoter-binding Protein, HsfA, from *M. xanthus*

*M. xanthus* DZ1F1 cells were grown exponentially (up to Klett units of ~80) in 1 liter of CYE liquid medium at 30 °C and heat-shocked at 42 °C for 20 min. Heat-shocked cells were prepared from total 10-liter cultures. AS fraction was prepared as described above except that the 2 μg of precipitates obtained after ammonium sulfate precipitation were suspended in TGED buffer (10 mM Tris-HCl (pH 8.0), 10% glycerol, 0.1 mM EDTA, 0.1 mM diithiothreitol, protease inhibitors (Roche)). AS fraction was applied to a DEAE-Sepharose column (Amersham Biosciences, Inc.) equilibrated with TGED buffer. HsfA was eluted with TGED buffer containing 0.1 M KCl (TGED0.1K). The activity of HsfA was detected by in vitro transcription analysis. The activity was applied to a heparin-agarose column (Amersham Biosciences, Inc.) equilibrated with TGED0.1K containing 8M urea. The sample was dialyzed against TGED0.1K containing 4 μM urea, TGED0.1K containing 2 μM urea, and TGED0.1K. The sample was cleared by centrifugation. The supernatant was applied to a DEAE-Sepharose column equilibrated with TGED0.1K. After washed with TGED0.1K, the column was eluted in the stepwise manner. HsfB was eluted with TGED0.3K.

The complex was applied to a heparin-agarose column equilibrated with TGED0.3K. This eluate was diluted with 2 volumes of TGED and applied to a heparin-agarose column equilibrated with TGED0.1K. The column was washed with TGED0.1K and stepwise eluted. RNAP was eluted with TGED0.5K. The eluate was diluted with 4 volumes of TGED and applied to a DNA-cellulose column (Amersham Biosciences, Inc.). The column was washed with TGED0.1K and stepwise eluted. RNAP containing SigA was eluted with TGED0.5K.

Purification of Recombinant Proteins

HsfA—The hsfA gene was cloned in pET24b (Novagen) (designated pET24b-hsfA). pET24b-hsfA was transformed into E. coli BL21(DE3), and the hsfA gene was overexpressed with 1 μM isopropyl-1-thio-B-D-galactopyranoside. Cells were harvested by centrifugation, suspended in TGED0.1K, and disrupted by sonication. The lysate was cleared by centrifugation. The lysate was applied to a DEAE-Sepharose column. The rest of purification procedure is described above.

HsfB—The hsfB gene was overexpressed as described above. Because HsfB was produced as inclusion bodies, the precipitates from the lysate were centrifuged to remove precipitates. The precipitates were then resuspended in TGED0.1K containing 8 μM urea. The sample was dialyzed against TGED0.1K containing 4 μM urea, TGED0.1K containing 2 μM urea, and TGED0.1K. The sample was cleared by centrifugation. The supernatant was applied to a DEAE-Sepharose column equilibrated with TGED0.1K. After washed with TGED0.1K, the column was eluted in the stepwise manner. HsfB was eluted with TGED0.3K.

The HsfB kinase domain (Met172 to Pro207) and the HsfB receiver domain (initiation Met to Gln277) were purified as described for HsfA.

In Vitro Phosphorylation

An in vitro phosphorylation reaction was performed in kination buffer (25 mM Tris-HCl (pH 7.4), 10 mM MgCl2, 5 mM 2-mercaptoethanol, 0.2 μM [γ-32P]ATP). 10 μM HsfA and 10 μM HsfB were incubated in 20 μl of the kination buffer at 30 °C. The reaction was stopped by adding 5 μl of stop solution (10% SDS, 0.4 M Tris-HCl (pH 6.8), 50 mM glycerol, 0.1 M 2-mercaptoethanol, 0.02% bromphenol blue). The samples were subjected to 15% SDS-PAGE and analyzed by autoradiography.

RESULTS

Identification of a Heat-shock Gene in *M. xanthus*—We first attempted to identify a heat-shock gene from *M. xanthus* which could be used for in vitro transcription analysis because no heat-shock gene had been identified. For this purpose, primer extension analysis was carried out by using specific primers to lonV (25), lonD (16, 17), two clpP (accession no. AF013216 and AF127082), and clpX (accession no. AF127082) genes because they are known to be heat-shock genes in other bacteria. It was found that only the lonD gene was induced by heat-shock (Fig. 1A, data not shown for other genes). Thus the promoter region for the lonD gene was assigned as TTGCGA for the −35 region and TACGTT for the −10 region as shown in Fig. 1B. These promoter sequences are more closely related to the E. coli ς70 recognition consensus sequences, TTGCGA (−35) and TATAAT (−10) (26, 27) than the ς32 CTTGAAA (−35) and CCCCATT (−10) and the ς24 GAACTT (−35) and TCTGA (−10) (2, 5) (Fig. 1C). Furthermore, the promoter sequences of the lonD gene not only at the −35/−10 regions but also surrounding regions, are very similar to those of the vegA gene, which is expressed during vegetative growth (28) (Fig. 1C). The vegA promoter has been shown to be recognized in vitro by RNAP containing SigA, the major housekeeping sigma factor in *M. xanthus* (29).
contrast to those heat-shock genes whose expressions are negatively regulated in some bacteria, the lonD promoter region does not contain such inverted repeat sequences (4).

**In Vitro Transcription Analysis**—To elucidate the mechanism regulating lonD expression, in vitro transcription analysis was performed. At first, the cytoplasmic fraction was fractionated by ammonium sulfate, and the fraction obtained between 40 and 65% was found to contain most of the lonD transcription activity (data not shown). This fraction is referred to as AS fraction. AS fraction was prepared either from heat-shocked cells (H-AS) or non-heat-shocked cells (NH-AS). Two different templates were constructed to distinguish the effect of heat-shock on the upstream region from the −35/−10 promoter regions. One template (plonD41) contains the promoter region up to −41 with respect to the transcription initiation site, and the other (plonD346) up to −346.

When plonD41 was used as a template, H-AS was more active than NH-AS (Fig. 2, lanes 2 and 3). This indicates that there may be a heat-shock sigma factor. This will be discussed further later. When plonD346 was used as a template, transcripts produced by H-AS increased compared with those by NH-AS (Fig. 2, lanes 5 and 6). These results revealed that a part of activation of lonD transcription was dependent on the upstream region (Fig. 2, lanes 3 and 6). Interestingly, this activation was restored when NH-AS was incubated at 42 °C for 10 min before the transcription reaction (lanes 4 and 7). Plasmids plonD41 (lanes 2–4) and plonD346 (lanes 5–7) were used as templates. Transcripts were analyzed by primer extension with the same primer used in Fig. 1A. Lane 1 represents products with in vivo total RNA prepared from heat-shocked cells as a control.

**Identification of the DNA Binding Site**—Because activation of lonD expression in vitro was dependent on the upstream region, next we attempted to identify possible trans-acting factors by DNA binding assay. The DNA fragment containing the region from −42 to −346 with respect to the transcription initiation site was used as a probe. As shown in Fig. 3A, there were retarded bands with all extracts, NH-AS (lane 1), H-AS (lane 2), and in vitro heat-shocked NH-AS (lane 3). This result suggests that DNA-binding protein(s) involved in lonD expression exist(s) in cells before heat-shock and supports the results shown in Fig. 2, in that lonD expression with NH-AS was activated by in vitro heat-shock. Furthermore, the DNA binding activity is apparently same before and after heat-shock, suggesting that the regulatory mechanism of the DNA binding activity is different from that of eukaryotic heat-shock factor.

To narrow the binding site(s), four other DNA fragments were used as shown in Fig. 3, B–E. The results indicate that the binding site(s) is (are) located between the NarI site (−140) and

**Fig. 1. Identification of lonD as a heat-shock gene. Panel A, primer extension analysis. Total RNA from cells before and after heat-shock was used. 32P-Labeled oligonucleotide 5′-CTCGAGCGGAGCCCTTC-3′ was used as a primer. Lanes G, A, T, and C represent sequence ladders. Lanes − and + represent transcripts before and after heat-shock, respectively. Panel B, the promoter region of the lonD gene. The transcription initiation site is indicated by +1 with a bold letter. The translation initiation codon is indicated by Met. Promoter sequences corresponding to the −10/−35 regions are underlined. The region protected in footprinting experiment is double underlined. The sites of restriction enzymes used in this study are indicated. Panel C, comparison of the lonD promoter sequence with the vegA promoter sequence of M. xanthus and α3, α5, and α3 consensus promoter sequences of E. coli. The 5′-TG-3′ element is indicated by a square.**

**Fig. 2. In vitro transcription analysis. AS fractions (lanes 2–7) were used as a source of protein components. Fractions were prepared from non-heat-shocked cells (lanes 2 and 5) and heat-shocked cells (lanes 3 and 6). In vitro heat-shock of NH-AS was performed at 42 °C for 10 min before transcription reaction (lanes 4 and 7). Plasmids plonD41 (lanes 2–4) and plonD346 (lanes 5–7) were used as templates. Transcripts were analyzed by primer extension with the same primer used in Fig. 1A. Lane 1 represents products with in vivo total RNA prepared from heat-shocked cells as a control.**
the BspEI site (−100).

We attempted to determine the sequences recognized by the DNA-binding protein(s) by footprinting analysis. As a probe, the HindII (−250)/BamHI (−42) fragment (probe B in Fig. 3) was labeled with [α-32P]dCTP by Klenow fragment. Note that EcoRI and BamHI are located in the vector. A5 fractions were used as a source of proteins. Lane 1, no fraction; lane 2, NH-AS; lane 3, H-AS; lane 4, in vitro heat-shocked NH-AS. Bound probes are indicated by arrows. Panel F, footprinting analysis. The probe used in panel B was used for DNA binding reactions. Lane 1 represents footprints from free probe. Lanes 2–4 represent footprints from probes bound with NH-AS, H-AS, and in vitro heat-shocked NH-AS, respectively. Lanes G, A, T, and C represent sequence ladders generated by primer 5'-GATCGTGCGTTTTTCCGCCCCCCGTC-3'.

**Fig. 3. Identification of the binding sites.** Panels A–E, DNA binding assay. Probes were prepared by digestion of plonD(346–42) with EcoRI and BamHI (panel A), HindII and BamHI (panel B), NarI and BamHI (panel C), BspEI and BamHI (panel D), and EcoRI and NarI (panel E) and by labeling with [α-32P]dCTP by Klenow fragment. Note that EcoRI and BamHI are located in the vector. A5 fractions were used as a source of proteins. Lane 1, no fraction; lane 2, NH-AS; lane 3, H-AS; lane 4, in vitro heat-shocked NH-AS. Bound probes are indicated by arrows. Panel F, footprinting analysis. The probe used in panel B was used for DNA binding reactions. Lane 1 represents footprints from free probe. Lanes 2–4 represent footprints from probes bound with NH-AS, H-AS, and in vitro heat-shocked NH-AS, respectively. Lanes G, A, T, and C represent sequence ladders generated by primer 5'-GATCGTGCGTTTTTCCGCCCCCCGTC-3'.

**Fig. 4. Mutational analysis.** Panel A, sequences of the DNA binding site. The mutated sequences are shown below the wild-type sequences. Panel B, DNA binding assay. Probes (−250 to approximately −100) containing the wild-type and mutated sequences were used. Bound probes are indicated by an arrow. − and + represent the absence and presence of H-AS, respectively. Panel C, in vitro transcription analysis. Template DNA containing either the wild-type or mutated sequences in the promoter region (−346 to approximately +95) and the wild-type sequence (−41 to approximately +95) was used with H-AS for in vitro transcription reaction. Panel D, in vivo expression. Total RNA was prepared from the parent strain (DZF1) and strains harboring the vector without the lonD promoter, with the wild-type lonD promoter (−346 to approximately +95 and −41 to approximately +95), and with the mutated lonD promoter. − and + represent non-heat-shocked cells and heat-shocked cells at 42 °C for 10 min, respectively. Transcripts were analyzed by primer extension analysis. The sequence of the primer used in this experiment is 5'-GGCGCCCGCGTCAGATG-3', which is specific to the sequence downstream of the cloned lonD promoter in the vector pSI1403Kmatt.

The plasmid pSI1403Kmattp was constructed by cloning the DNA fragment containing attP into pSI1403Km (32). Those plasmids were then integrated into the attB site of chromosomal DNA of *M. xanthus* DZF1 by electroporation. The integration of the plasmid at attB was confirmed by Southern blot analysis (data not shown). The antisense oligonucleotide that is specific to the sequence downstream of the cloned lonD promoter in pSI1403Kmatt was used as a primer so that only transcripts from the integrated promoter were detected by primer extension analysis. As shown in Fig. 4D, heat-shock induction of lonD expression was observed only in the strain harboring the promoter containing the wild-type sequences with the upstream region. These results demonstrate that the upstream region centered at −114.5 is required for the heat-shock induction of lonD expression both in vivo and in vitro.

Purification and Identification of a DNA-Binding Protein Specific to the lonD Promoter—Next the lonD promoter-binding protein was purified by ammonium sulfate fractionation followed by chromatography including DEAE-Sepharose, heparin-agarose, and DNA affinity containing the lonD promoter from −128 to −105. The activity of the protein was checked by DNA binding assay. After the second DNA affinity column chromatography, a protein with an apparent molecular mass of 55 kDa was obtained (Fig. 5).

To clone the gene encoding the lonD promoter-binding protein, the amino acid sequence of the N terminus of the purified protein was determined and found to be MNQVKRAKLVLVVD-DSVVLKAVTQILQREG. From this sequence, two degenerated oligonucleotides were designed, and PCR amplification was carried out by using the oligonucleotides as primers with *M. xanthus* chromosomal DNA as template. Southern blot analysis was performed using PCR-amplified products as a probe with XmaI digests of *M. xanthus* chromosomal DNA. A 1.6-kb DNA fragment from the XmaI digests which hybridized with the probe was cloned. Analysis of the DNA sequence revealed that the DNA fragment contained a gene encoding an ORF (485
HsfB was autophosphorylated with these purified proteins. As found in other histidine kinases, DEAE-Sepharose chromatography pressed in heparin-agarose, and DNA affinity. HsfB was also overexpressed and purified by chromatography including DEAE-Sepharose, heparin-agarose, and DNA affinity. HsfB was also overexpressed and purified by chromatography including DEAE-Sepharose, heparin-agarose, and DNA affinity. HsfB was also overexpressed and purified by chromatography including DEAE-Sepharose, heparin-agarose, and DNA affinity. HsfB was also overexpressed and purified by chromatography including DEAE-Sepharose, heparin-agarose, and DNA affinity. HsfB was also overexpressed and purified by chromatography including DEAE-Sepharose, heparin-agarose, and DNA affinity.

Another ORF was found immediately upstream of hsfA. ORF shows high sequence similarity to histidine kinases of the NtrC family proteins (33–35). However, phosphorylation of HsfB-R was not observed with either HsfB (Fig. 6, lane 2) and be able to phosphorylate HsfA (Fig. 6, lane 8). However, phosphorylation of HsfB-R was not observed with either HsfB (Fig. 6, lane 5) or HsfB-K (Fig. 6, lane 7). These results suggest that another histidine kinase may exist which phosphorylates the receiver domain of HsfB in M. xanthus. Such phosphorylation of the receiver domain of HsfB may modulate the function of the HsfB.

Transcriptional Activation of lonD by HsfA and HsfB in Vitro—To confirm that HsfA expressed in and purified from E. coli cells binds the sequences recognized by SigA (29), we examined whether or not HsfA from M. xanthus and HsfA from E. coli bound sequences identical to those determined by AS fractions in Fig. 3F.

The enhancer-binding proteins usually activate transcription with RNAP containing RpoN (σN, σ24) (33–35) except for one case reported thus far (39). The sequences of the lonD promoter are compared with those of σ24-like promoters of M. xanthus genes, P4251 (40), mbaA (41), and pilA (42) and E. coli σ24 consensus sequences (43) (Fig. 7B). Although the−12 region of the lonD promoter shows some similarity to σ24-like promoters, the−24 region does not show any similarity. It has been demonstrated by mutational analysis that bases G, A, and G of the P4251 promoter at positions −26, −24, and −22, respectively, are important for its transcription (40), and these bases are conserved among these M. xanthus σ24-like promoters (Fig. 7B). Therefore, it is unlikely that the lonD promoter is recognized by RpoN. In contrast, because the lonD promoter is similar to the uga promoter (Fig. 1C), which has been shown to be recognized by SigA (29), we examined whether or not RNAP/SigA can transcribe the lonD gene in vitro. First, RNAP/SigA was purified from M. xanthus cells by ammonium sulfate fractionation followed by chromatography including DEAE-Sepharose, heparin-agarose, and DNA-cellulose. This preparation was used in transcription reactions performed in vitro with template DNA containing the lonD promoter region. RNAP/SigA was indeed able to initiate transcription in vitro (Fig. 7C, organization is also found in AsgA (37) and AsgD (38) of M. xanthus. AsgA has been shown to have autophosphorylation activity in vitro, but phosphotransfer to its own receiver domain was not observed. Similar biochemical assays have not been performed for AsgD. Because the receiver domain of HsfB is highly homologous to other response regulators, we next examined whether the receiver domain of HsfB (HsfB-R) is phosphorylated in vitro by the kinase domain of HsfB (HsfB-K). Thus, HsfB-K and HsfB-R were overexpressed in E. coli and purified from inclusion bodies followed by DEAE-Sepharose chromatography. HsfB-K was found to be autophosphorylated (Fig. 6, lane 2) and be able to phosphorylate HsfA (Fig. 6, lane 8). However, phosphorylation of HsfB-R was not observed with either HsfB (Fig. 6, lane 5) or HsfB-K (Fig. 6, lane 7).
lane 2) from the same initiation site as found in vivo (Fig. 7C, lane 1). Enhancer-binding proteins with receiver domains are activated for the transcriptional activity by phosphorylation with cognate histidine kinases. When in vitro transcription reactions were performed with HsfA purified from E. coli and RNAP/SigA, transcriptional activation was observed (Fig. 7C, lane 3). When HsfA was mixed with HsfB purified from E. coli before the in vitro transcription reactions, more activation was observed (Fig. 7C, lane 4). Therefore, HsfA activated in vitro transcription of lonD with RNAP/SigA in a manner dependent on phosphorylation by HsfB. It is likely that the activation observed in the absence of HsfB was the result of HsfA partially phosphorylated by E. coli histidine kinases and/or low molecular weight phosphodonors such as acetyl phosphate during overexpression in E. coli. It should be noted that HsfB and HsfA could not activate RNAP/SigA when the vegA promoter was used as template for in vitro transcription (data not shown).

The hsfB and hsfA Genes Are Required for Normal Vegetative Growth—We attempted to construct deletion mutants of hsfA and hsfB genes to elucidate their functions in vivo. When the linearized plasmid in which the hsfB gene was replaced by a kanamycin-resistant gene was electroporated into M. xanthus DZF1, no transformants were obtained (data not shown). To determine whether this result was caused by the absence of the functional hsfB gene, the plasmid containing the hsfB gene was first integrated at the attB site of M. xanthus DZF1 chromosome. In this case, the original hsfB gene could be replaced by the kanamycin-resistant gene (data not shown). This result indicates that the hsfB gene is essential for normal vegetative growth. Furthermore, the same strategy was performed for the hsfA gene, and it was also found that the hsfA gene was required for normal vegetative growth (data not shown).

**DISCUSSION**

We have identified a new two-component His-Asp phosphorelay system that regulates expression of a heat-shock gene, lonD, in M. xanthus. This system consists of a response regulator, HsfA, and a hybrid histidine kinase, HsfB. HsfA also belongs to the NtrC family or enhancer-binding proteins. HsfB has a rather unusual domain organization and consists of a receiver domain at the N-terminal end and a kinase domain at the C-terminal end (Fig. 8).

Enhancer-binding proteins activate RNAP containing RpoN by binding promoter regions that are localized in tandem at >100 bp upstream with respect to the transcription initiation site (33–35). Enhancer-binding proteins have ATPase activity that is required for transcriptional activation. A subgroup of enhancer-binding proteins including NtrC belongs to response regulators of the two-component His-Asp phosphorelay signal transduction system. Therefore, it is regulated by a cognate sensor kinase, NtrB, in the case of NtrC. Although DNA binding of NtrC can be achieved in the absence of phosphorylation, only the phosphorylated form of NtrC is able to promote transcription. Phosphorylation is necessary for ATPase activity, oligomerization, and formation of an open transcriptional complex.

We have demonstrated that extracts from both non-heat-shocked cells and heat-shocked cells possess DNA binding activity to the upstream promoter region of lonD, but that only the extract from heat-shocked cells is able to activate transcription in a manner dependent on the upstream promoter region of lonD. It was also found that transcriptional activity in the extract from non-heat-shocked cells is restored by in vitro heat treatment at 42 °C before transcription reaction, suggesting that a factor(s) in the extract can sense temperature shift and activate transcription of lonD.

Furthermore, we showed that HsfA is phosphorylated in vitro by HsfB and that transcription of lonD is activated in the presence of HsfA and HsfB. Although the extract prepared from non-heat-shocked cells can become transcriptionally active after in vitro heat-shock, purified HsfB was not activated by elevated temperature (data not shown). This suggests that HsfB is not the sensor kinase directly sensing heat-shock (this will be discussed later in detail). As mentioned above, two binding sites are usually found in the upstream promoter region of genes regulated by enhancer-binding proteins, whereas only one region (−122 to −107) was identified in the lonD promoter by DNA binding assay. When this region was mutated, no DNA binding activity was observed, and transcriptional activation was abolished both in vivo and in vitro. However, footprinting analysis exhibited another protected region. The latter region may be bound by HsfA only when it and the former region exist together. It is known that the enhancer-binding proteins typically activate transcription with RNAP containing RpoN. However, we performed in vitro transcription with the major sigma factor, SigA, because the lonD promoter regions show higher similarity to σ70 consensus sequences than σ54 (Figs. 1C and 7B). It was found that HsfA promoted transcription of lonD in vitro with RNAP/SigA. It has been reported that Rhodobacter capsulatus NtrC activates RNAP containing
RpoD or 70, the housekeeping sigma factor (39).

Although the upstream promoter region was shown to be necessary for in vivo heat-shock induction of lonD, in vitro transcription analysis showed an increase of transcripts by heat-shock in vitro without the upstream promoter region of lonD. Because the −35/−10 promoter regions are recognized by RNAP/SigA, this increase may result from stabilization and/or modification of SigA in H-AS. RNAP/SigA in H-AS may be more active for the lonD promoter because no difference by heat-shock was observed for in vitro transcription with the vegA promoter. Furthermore, it is possible that the 5′-TG-3′ sequence element located 1 base upstream from the −10 hexamer element of the lonD promoter (indicated by a square in Fig. 1C) provides a motif necessary for transcription initiation as found in some promoters of E. coli (44). This 5′-TG-3′ element is not found in the vegA promoter. Therefore, this element may contribute to the increase of transcripts for lonD by heat-shock in vitro in the absence of the upstream promoter region and the HsfA/HsfB system. It has been demonstrated that some of E. coli promoters are recognized in vitro by both RNA polymerase containing RpoD and RpoS, the stationary phase sigma factor (σ32, σ38) (45). In addition, the increase in RpoS level is observed in E. coli when cells are exposed to heat-shock (46). Therefore, it is possible that the M. xanthus stationary phase sigma factor, SigD (11), may be increased by heat-shock, and SigD may be able to recognize the lonD promoter in vitro, resulting in the increase of transcripts with H-AS in the absence of the upstream promoter and the HsfA/HsfB system.

The His-Asp phosphorelay signal transduction system consists of two basic components, a sensor kinase and a response regulator. However, recent studies have demonstrated that three or four components (or domains) are utilized in one phosphorelay signal transduction event in which a phosphate group is transferred from histidine to aspartate to histidine to aspartate (His-Asp-His-Asp phosphorelay) (47). The first known phosphorelay was reported for regulation of initiation of sporulation in B. subtilis (48). This relay begins with autophosphorylation of one of three sensor kinases, KinA, KinB, or KinC. The phosphate group is then transferred to a response regulator, Spo0F. Spo0F serves as a phosphodonor for Spo0B. Finally, the phosphate group is passed from Spo0B to Spo0A, which regulates a number of genes involved in sporulation.

Hybrid kinases with transmitter domains of sensor kinases and receiver domains of response regulators are usually found in His-Asp-His-Asp phosphorelay. A receiver domain is typically fused to a transmitter domain at the C terminus in hybrid kinases, but it also can be found at the N terminus as found in M. xanthus HsfA (this study), AsgA (37), and AsgD (38). In Saccharomyces cerevisiae osmoregulation is controlled by the Sn1p-Ypd1p-Ssk1p system (49). The first two phosphorylation sites are located in the transmembrane hybrid kinase Sn1p. From Sn1p the phosphate group is transferred to Ypd1p, then to Ssk1p. Ssk1p modulates the downstream MAP kinase cascade. The BvgS-BvgA two-component system modulates regulation of virulence factors in Bordetella pertussis (50). In this system the first three steps of the four-step phosphorelay occur within the single protein BvgS. This pathway represents another organizational design. Furthermore, Ralstonia solanacearum utilizes in control of expression of virulence factors the PheS-PheR-OrfQ system in which the second and third phosphorylation sites appear to be located in the hybrid kinase PheR (51). It should be noted that PheR has the same domain organization as HsfB. With these instances, it is tempting to speculate that there may exist a presently unidentified kinase (HsfX in Fig. 8) which functions as a temperature sensor and activates HsfB by phosphorylation of the receiver domain upon heat-shock because purified HsfB is not able to be activated by temperature shift in vitro and contains a receiver domain at the N terminus. However, we cannot exclude possibility that a factor(s) other than a sensor kinase may sense heat-shock and transduce signals to HsfB. For example, in control of nitrogen assimilation by the NtrB/NtrC system, protein II or PII functions as a sensory component responsible for sensing 2-keto glutarate whose concentration reflects changes in the nitrogen status of the cell (52).

The present HsfA/HsfB system is the two-component His-Asp phosphorelay signal transduction system required for activation of a heat-shock gene and is also necessary for vegetative growth. Because lonD is dispensable for vegetative growth in M. xanthus (16, 17), the HsfA/HsfB system appears to regulate genes required for vegetative growth in addition to developmental genes such as lonD, an essential gene for fruiting body development (16, 17).
Acknowledgments—We are grateful to M. Inouye for discussion and preparation of this manuscript. We thank L. Vales for critical reading of this manuscript. We also thank C. Xu for DNA manipulation and K. Yamanaka for suggestions.

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Transcriptional Activation of a Heat-shock Gene, lonD, of Myxococcus xanthus by a Two Component Histidine-Aspartate Phosphorelay System

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J. Biol. Chem. 2002, 277:6170-6177.
doi: 10.1074/jbc.M110155200 originally published online December 17, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M110155200

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