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Characterization of the Genes and Proteins of a Two-Component System from the Hyperthermophilic Bacterium *Thermotoga maritima*

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As a step towards studying representative members of the two-component family of signal transduction proteins, we have cloned genes encoding a histidine protein kinase and a response regulator from the hyperthermophilic bacterium *Thermotoga maritima*. The genes have been designated *hpkA* and *drrA*, respectively. The deduced HpkA sequence contains all five characteristic histidine protein kinase motifs with the same relative order and spacing found in the mesophilic bacterial proteins. A hydropathy profile indicates that HpkA possesses only one membrane-spanning segment located at the extreme N terminus. The N-terminal region of DrrA exhibits all of the characteristics of the conserved domains of mesophilic bacterial response regulators, and the C-terminal region shows high similarity to the OmpR-PhoB subfamily of DNA-binding proteins. Recombinant *T. maritima* proteins, truncated HpkA lacking the putative membrane-spanning N-terminal amino acids and DrrA, were expressed in *Escherichia coli*. Partial purification of *T. maritima* proteins was achieved by heat denaturation of *E. coli* host proteins. In an in vitro assay, truncated HpkA protein was autophosphorylated in the presence of ATP. Thus, the N-terminal hydrophobic region is not required for kinase activity. Phosphotransfer between truncated HpkA and DrrA was demonstrated in vitro with the partially purified proteins. The phosphorylation reactions were strongly temperature dependent. The results indicate that the recombinant *T. maritima* two-component proteins overexpressed in *E. coli* are stable as well as enzymatically active at elevated temperatures.

Bacteria live in an environment in which they encounter rapid and unexpected changes. In order to effectively compete and survive, they continuously monitor external conditions and respond accordingly. Many bacterial regulatory systems that mediate such adaptive responses utilize a conserved signaling strategy involving histidine protein kinases and response regulator proteins, commonly referred to as two-component systems (for reviews, see references 15 and 26). In these signaling pathways, the histidine protein kinase autophosphorylates at a specific histidine residue, creating a high-energy phosphoryl group that is transferred to an aspartic acid residue of the response regulator protein. Phosphorylation of the response regulator, in most cases, creates an active form of the protein, capable of eliciting the output response. There is much variation in the specific arrangements of conserved domains within the proteins and further variation in the way the protein components are organized into specific signaling pathways, often interacting with numerous and diverse auxiliary proteins. Still, structural and functional studies of the conserved domains, such as the response regulator domain represented by CheY, have elucidated general features applicable to most members of the family (25, 32). At the present time, structural information has not been obtained for either the conserved histidine protein kinase domain or an intact multidomain response regulator protein.

Thermophilic bacteria, such as *Thermotoga maritima*, which inhabits an extreme environment and has an optimal growth temperature of 80°C (5), are potential sources for proteins of unusually high thermostability. The inherent stability of proteins from thermophiles provides advantages for structural analysis (17). Despite necessary adaptations to allow function at elevated temperatures, many proteins from thermophiles have high sequence similarity with homologs from mesophiles (1). The existence of histidine protein kinases and response regulator proteins in thermophilic bacteria (6, 30) is not unexpected, given the widespread occurrence of two-component systems throughout the bacterial kingdom and in eukaryotes as well (29). In order to further structure-function analyses of these proteins, a search was conducted for a representative member of the histidine protein kinase family encoded by *T. maritima* genomic DNA. PCR amplification using degenerate oligonucleotides with sequences derived from conserved regions of the histidine protein kinase family produced a fragment with sequence similarity to a region of the histidine protein kinases. This PCR fragment was used in hybridization studies to identify a clone containing a histidine protein kinase gene and surrounding sequences encoding a response regulator protein and the N-terminal region of a thymidine-pyrimidine nucleoside phosphorylase homolog.

The products of the *T. maritima* genes showed significant sequence similarity to a number of histidine protein kinase-response regulator pairs, with greatest similarity to members of the phosphate assimilation pathway (PhoR-PhoB) (11, 12), the vancomycin resistance regulators (VanS-VanR) (2), and the regulators of aerobic and anaerobic respiration gene expression (ResE-ResD) (23, 28). The *T. maritima* genes have been designated *hpkA* and *drrA*, encoding a histidine protein kinase and DNA-binding response regulator, respectively. Sequence analysis of HpkA indicated a histidine protein kinase with a

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single membrane-spanning region. DrrA was found to contain a characteristic N-terminal regulatory domain and a C-terminal effector domain belonging to the OmpR-PhoB subfamily of transcriptional regulators (24). The HpkA and DrrA proteins, expressed in *Escherichia coli* and partially purified from heat-denatured cell lysates, exhibited autophosphorylation and phosphotransfer activities consistent with their putative roles in two-component signal transduction.

MATERIALS AND METHODS

DNA manipulation and sequence analyses. Molecular cloning and manipulation of plasmid DNA were done by standard methods (18). Restriction, ligation, and other DNA modification enzymes were purchased from New England Biolabs and Boehringer Mannheim. DNA sequencing was performed by the dideoxy chain termination method (19) with Sequenase 2.0 as recommended by the manufacturer, United States Biochemical. The sequence reported in Fig. 1B was determined on both strands with nucleotide mixtures containing dGTP and 7-deaza-dGTP in separate sequencing reactions to eliminate compression artifacts. DNA and protein sequences were analyzed and manipulated by using programs in the University of Wisconsin Genetics Computer Group software suite (3).

Bacterial strains and growth conditions. *E. coli* DH5α, used as host for plasmids, was grown overnight at 37°C in Luria-Bertani (LB) broth or on LB agar containing 100 μg of ampicillin per ml. For expression of *T. maritima* proteins, E507 (27), which contains a T7 RNA polymerase gene under control of an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible *lacUV5* promoter, served as host for plasmids derived from pJES307 (31), with the genes of interest inserted downstream of the T7 promoter. E507 cells containing pJES307 derivatives were grown to an A_{600} of 0.5 in 1.5 liters of LB broth with 100 μg of ampicillin per ml, and protein expression was induced by addition of IPTG to a final concentration of 0.5 mM. After 2 h of induction, cells were harvested.

PCR. From the best alignment of mesophilic histidine protein kinase sequences, a pair of degenerate oligonucleotides was designed and used for PCR primers, 5′-TC(A/T)CA(T/C)GA(A/G)AT(T/C/A)AA(A/G)AA(T/C)CC-3′ and 5′-GG(A/G)AT(A/T)CC(A/T)GG(A/T)CC(A/G)T(T/C)(A/G)TC-3′, corresponding to coding and anticoding strands, respectively. The oligonucleotides were synthesized (UMDNJ Biochemistry Department Oligonucleotide Synthesis Facility) with two different restriction sites, *Hin*dIII at the 5′ end of the coding strand and *Eco*RI at the 5′ end of the anticoding strand. PCR, using these two oligonucleotides (100 pmol) as primers and *T. maritima* genomic DNA (0.5 μg), obtained from Janet Westpheling, University of Georgia, as template, was performed in a 30-cycle reaction in an MJ Research Minicycler with native *Pfu* polymerase (Stratagene) in its supplied reaction buffer. The initial annealing temperature of 40°C in PCR was shifted after the fifth cycle to 50°C and was maintained for the remaining 25 cycles. Following visualization on ethidium bromide-stained agarose gels, the PCR products corresponding to fragments of approximately 460 bp were excised. The purified PCR fragments were digested with *Hin*dIII and *Eco*RI and separated on a preparative agarose gel. The fragments were excised, purified, and ligated to *Hin*dIII-*Eco*RI-digested pBluescript II KS(−) vectors (Stratagene). The resulting plasmids were transformed into competent DH5α cells. Plasmid DNA was isolated and sequenced.

Southern blot analysis. *T. maritima* genomic DNA was digested with appropriate restriction enzymes. The digests were separated on a 1% agarose gel and blotted onto a Nytran nylon membrane via the alkaline transfer method in a Transblotter system according to the manufacturer's recommendations (Schleicher & Schuell). Hybridization was performed with a Rad-Free probe labeling and detection system (Schleicher & Schuell). Positive clones were selected by hybridization with a biotin-labeled probe generated from a PCR fragment of 460 bp containing sequences homologous to the mesophilic histidine protein kinase genes.

Library construction and colony hybridization. The first minilibrary was constructed from *Bam*HI-*Hin*dIII fragments generated by digestion of *T. maritima* genomic DNA. Fragments ranging from 2.5 to 6 kb were purified from an agarose gel and ligated to *Bam*HI-*Hin*dIII-digested, dephosphorylated pBluescript II KS(−) vectors. This library was used to transform *E. coli* DH5α cells. Ampicillin-resistant colonies present after 18 h of growth on LB agar were transferred to Nytran nylon membranes. Hybridization was performed as described for Southern blot analysis. A similar approach was used for the second minilibrary, which was constructed from 0.5- to 2-kb *Eco*RI genomic DNA fragments.

Construction of plasmids expressing HpkA and DrrA. PCR was used to amplify regions encoding the *T. maritima hpkA* and *drrA* genes with *Nde*I and *Hin*dIII sites added at the 5′ ends of the coding and anticoding strands, respectively. After digestion with appropriate enzymes, PCR fragments were ligated to *Nde*I-*Hin*dIII-digested pJES307 vectors. The resulting plasmids, pJL9B containing full-length *drrA* and pJL26R containing full-length *hpkA*, expressing the genes from T7 promoters, were transformed into *E. coli* DH5α and later into E507.

Four plasmids were constructed for expression of truncated HpkA proteins lacking 22, 47, 57, and 65 N-terminal amino acids. PCR amplification with appropriately designed oligonucleotides was used to generate *hpkA* coding regions with *Nde*I and *Hin*dIII sites added at the 5′ ends of the coding and anticoding strands, respectively. The fragments were ligated to *Nde*I-*Hin*dIII-digested pJES307 vectors to generate pJLR22, pJLR47, pJLR57, and pJLR65, and the resulting plasmids were transformed into *E. coli* DH5α. Following selection, the vector inserts were confirmed by DNA sequencing and the plasmids were transformed into *E. coli* E507.

Partial purification of HpkA and DrrA. Following growth, cells were harvested by centrifugation at 4,000 × *g* for 10 min at 4°C. The cell pellet was resuspended at 5 ml/g (wet weight) of cells in 25 mM Tris-HCl–50 mM KCl (pH 8.0), and cells were lysed by sonication. The cell lysate was centrifuged at 15,000 × *g* for 30 min at 4°C. The soluble fraction was diluted to approximately 20 mg of total protein per ml (estimated by absorbance, assuming that 1 mg of protein per ml has A_{280} = 1.0) and was heated for 10 min at 70°C. The majority of host proteins precipitated and were removed by centrifugation at 15,000 × *g* for 30 min at 4°C. The levels of expression and purification of the *T. maritima* proteins were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), and the concentrations of HpkA and DrrA proteins were estimated by the intensity of Coomassie blue stain with a bovine serum albumin standard of known concentration.

Phosphorylation activities of HpkA and DrrA. Heat-treated lysates were incubated with [γ-^{32}P]ATP (3,000 Ci/mmol, Amersham) in a total volume of 10 μl of TKME buffer (50 mM Tris-HCl, 50 mM KCl, 5 mM $MgCl_2$, 0.5 mM EDTA [pH 8.0]) containing 0.1 mM dithiothreitol and 0.1 mM [γ-^{32}P]ATP at a final specific activity of 5 Ci/mmol. After incubation for the indicated times at the indicated temperatures, reactions were stopped by addition of 10 μl of concentrated Laemmli SDS loading buffer (0.125 M Tris-HCl, 4% SDS, 20% glycerol, 0.004% bromophenol blue, 2.5% β-mercaptoethanol [pH 6.8]). The reaction products were applied without heating to 15% polyacrylamide gels. Following electrophoresis, the undried gels were exposed to Biomax film (Kodak) with an intensifying screen for approximately 4 h at −70°C.

Nucleotide sequence accession numbers. Nucleic acid sequences for the genes recorded herein were submitted to GenBank and given accession numbers U67196.

RESULTS AND DISCUSSION

Cloning of *hpkA* and *drrA* from *T. maritima*. Degenerate oligonucleotides designed from conserved regions of histidine protein kinases were used as primers for PCR amplification to identify putative genes encoding histidine protein kinase homologs in the hyperthermophilic bacterium *T. maritima*. The conserved histidine protein kinase domain can be considered to consist of two subdomains (15, 24). The first subdomain contains a conserved sequence motif, termed H box, that contains the site of histidine phosphorylation and is generally located N proximal to the second subdomain. Among several highly conserved sequences within the second subdomain, a region designated G1 box contains a conserved DXGXG motif thought to comprise a nucleotide binding site. The design of the degenerate oligonucleotides for PCR-mediated cloning was based on these two conserved regions in the histidine protein kinase family. The degree of degeneracy was decreased by eliminating some codons on the basis of the infrequency of codon usage in *T. maritima* (6). The degenerate primers were used in PCR amplification of *T. maritima* genomic DNA. The annealing temperature was reduced in the first five cycles to allow some mismatch within homologous sequences. DNA sequences were determined for the PCR amplification products with lengths of approximately 460 bp, the expected size for the product from a histidine protein kinase gene. The FASTA program (16) was used to search for similarity of these translated sequences with a library of histidine protein kinase sequences as well as the rest of the protein database. The protein sequence predicted from one of the PCR fragments showed similarity to a region in the histidine protein kinases with highest similarity (32 to 37% identity) to PhoR protein sequences (10, 12, 22). Other PCR fragments had sequence similarity with some transporters or DNA-binding proteins that were presumably amplified because of the DXGXG motif which is commonly found in such proteins. To obtain a clone of the complete histidine protein kinase gene, the PCR fragment

containing a partial sequence of the putative *T. maritima* histidine protein kinase was used as a probe for Southern blot analysis of several different restriction digests of *T. maritima* genomic DNA and a restriction map was constructed. With the expectation that the positioning of the response regulator and histidine protein kinase genes in an operon as observed in mesophilic bacteria might be conserved in *T. maritima*, a 4-kb *Bam*HI-*Hin*dIII fragment which extends mostly to the 5′ side of the region identified by PCR was targeted for cloning. An additional 1.1-kb *Eco*RI fragment which extends to the 3′ side of the region identified by PCR was also targeted for cloning. Two minilibraries each containing fragments of the desired sizes were constructed separately. The first minilibrary was constructed by ligating the *Bam*HI-*Hin*dIII-digested genomic DNA fragments in the size range of 2.5 to 6 kb to a pBluescript II KS(−) vector as described in Materials and Methods. A positive clone was isolated by colony hybridization using the 460-bp PCR fragment as a probe. Restriction mapping demonstrated that the insert consisted of approximately 2.3 kb of DNA. Subsequent analysis by restriction mapping and DNA sequence determination revealed preservation of the *Hin*dIII site at the 3′ cloning junction, but lack of the expected *Bam*HI site at the 5′ cloning end. The shorter fragment and loss of the *Bam*HI site may have resulted from additional cleavage within the genomic DNA prior to isolation or by processing within the *E. coli* host subsequent to transformation. To confirm that this 2.3-kb fragment represented a contiguous region of *T. maritima* genomic DNA, a pair of oligonucleotides with sequences corresponding to the 5′ and 3′ ends of this 2.3-kb fragment were used as primers for PCR amplification of *T. maritima* genomic DNA. No differences were observed between the size or restriction maps of the PCR product and those of the 2.3-kb fragment identified in the positive clone. Furthermore, the nucleotide sequences of the 5′ and 3′ regions of the PCR product were identical to those found in the positive clone. A second minilibrary was constructed by cloning the 0.5- to 2-kb *Eco*RI fragments into pBluescript II KS(−) vectors (see Materials and Methods). Sequence determination of the plasmid of a positive clone identified by a colony hybridization screen identified the 3′ end of the histidine protein kinase gene and its flanking sequence (Fig. 1).

The complete sequence determination of the isolated 2.3-kb fragment and the overlapping 1.1-kb *Eco*RI fragment revealed two complete open reading frames (ORFs) separated by 3 nucleotides and a partial ORF which was 5 nucleotides from the second complete ORF (Fig. 1A). Potential Shine-Dalgarno ribosome binding sequences (7) were identified at appropriate distances upstream of the putative initiation codons of all three ORFs (Fig. 1B). Two protein sequences were deduced from the two complete ORFs, each with a putative translational initiation codon (ATG) and a termination codon (TGA). A search of databases with FASTA revealed significant sequence similarity between the two deduced protein sequences and the members of the histidine protein kinase and response regulator families. The genes encoding the putative *T. maritima* two-component proteins were designated *hpkA* and *drrA*. A polypeptide of 137 amino acids is predicted from the sequence of the partial ORF with a putative start codon, GTG. A database search showed that the deduced polypeptide sequence had significant similarity to the N terminus of the thymidine-pyrimidine nucleoside phosphorylase family. Over the N-terminal 137 amino acids encoded by the ORF, the *T. maritima* protein is 54% identical to *Bacillus subtilis* pyrimidine nucleoside phosphorylase (20) and 49% identical to *E. coli* thymidine phosphorylase (33).

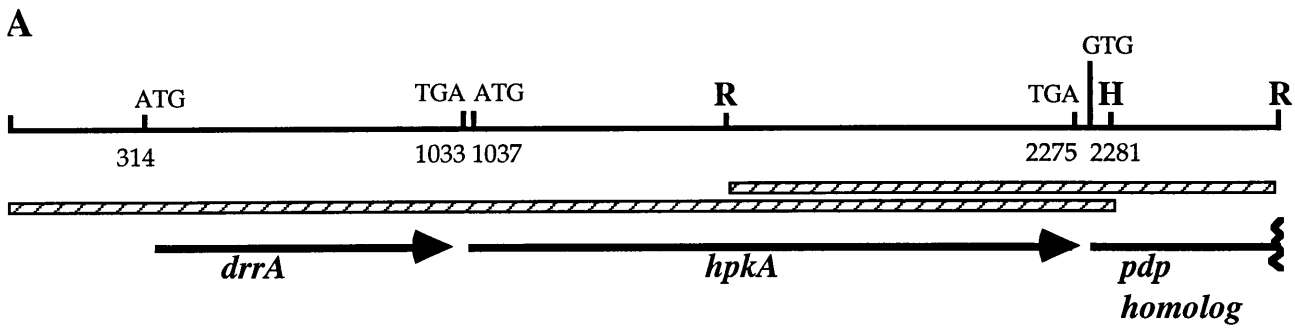
Similarity between *T. maritima* and mesophilic two-component proteins. The *T. maritima hpkA* gene encodes a protein of 412 amino acids with a calculated molecular mass of 48.0 kDa. This protein has a high content of charged residues and a calculated isoelectric point (pI) of 5. The deduced *T. maritima* histidine protein kinase sequence showed greatest similarity to a number of mesophilic PhoR proteins (36 to 38% identity) (10, 12, 22) (Fig. 2). Slightly lower levels of similarity were observed for *Caulobacter crescentus* PleC (34) and *B. subtilis* ResE (23) (30% identity).

The deduced HpkA sequence contains all features characteristic of the histidine protein kinase family. HpkA, like other histidine protein kinases, has a conserved histidine, presumably the site of phosphorylation, that precedes a conserved asparagine by approximately 100 residues. The kinase domain of *T. maritima* HpkA also contains four other highly conserved sequences termed the N, G1, F, and G2 boxes. Presumably, these motifs form a nucleotide binding surface within the active site. The hydrophobicity profile (8) of *T. maritima* HpkA suggests that amino acids 1 to 20 form a hydrophobic region sufficiently long to span the membrane once whereas most of the mesophilic membrane-associated histidine protein kinases possess two transmembrane regions.

The *T. maritima drrA* gene encodes a protein of 239 amino acids with a calculated molecular mass of 27.6 kDa and a calculated pI of 6. The predicted *T. maritima* response regulator sequence showed significant similarity to numerous mesophilic response regulator proteins. *B. subtilis* PhoP (9, 21) and YycF (14) showed the highest similarity, with 52% identity over their entire lengths (Fig. 3). Another 30 response regulator proteins showed 35 to 45% identity over the length of the *T. maritima* response regulator protein.

The response regulator superfamily is characterized by a conserved N-terminal regulatory domain of approximately 125 amino acids usually attached via a linker sequence to a C-terminal effector domain of variable size. Predictions of response regulator structure are based on the known three-dimensional structure of the chemotaxis response regulator, CheY, which consists of five parallel β strands surrounded by five amphipathic α helices. The protein sequence of *T. maritima* DrrA can be aligned with this secondary structure on the basis of residues that correspond to the hydrophobic core. Additionally, within the regulatory domain, the aspartates that form the active site and the nearby lysine are present in the *T. maritima* DrrA sequence. Response regulator sequences have been divided into five subfamilies according to homologies in their effector domains (24, 32). *B. subtilis* PhoP (9, 21), which showed the highest identity to *T. maritima* DrrA, belongs to the OmpR-PhoB subfamily. The C-terminal effector domain of *T. maritima* DrrA sequence exhibits most of the conserved residues present in the DNA-binding domain of OmpR-PhoB subfamily response regulators.

It is intriguing that sequence comparisons revealed three pairs of two-component systems in which both components exhibited high sequence similarity to the *T. maritima* proteins. One pair was the *B. subtilis* PhoR-PhoB proteins that regulate expression of genes involved in phosphate assimilation (11, 12). Another pair was the *Enterococcus faecium* VanS-VanR proteins that activate the expression of genes responsible for vancomycin resistance (2). The similarity between the Van and Pho proteins has been previously noted, and PhoB is the only response regulator aside from VanR that has been found to transfer phosphoryl groups from VanS, the histidine protein kinase (4). The third pair was the *B. subtilis* ResE-ResD proteins that are essential for aerobic and anaerobic respiration and are thought to have an indirect role in phosphate regula-



B

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1
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121
GGAGTTTAAACCGGAATCTCTCTTTGCGAACAAGATGATGATCTTCTTCTACGTGTCTCTTTTTCTGCTCTTTCTGTTTCTTTCTGCATGGTTCACTTTCAGGAAAAGATCCTATCAAAA
241                                                                                                    drrA
AACTAGTTCGTAAATGTTAAAATAGTATAGAAACCAGATCTGAAAGGAGGTGTACCCCCTCGGGGGATCGAAGATGGCGAAAAAGAAGATTCTGGTGGTTGACGACGACCCGGCAATTCT
                                                                                                       M  A  K  K  K  I  L  V  V  D  D  D  P  A  I  L
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M  R  A  Y  D  V  I  L  K  K  R  N  G  E  K  L  S  R  E  E  I  E  F  M  V  G  G  Y  V  K  G  E  I  P  D  Y  Q  M  A  A
2401
TTTTTGATGGCCGTTTACTTCCGCCATCTCGACGAGGAGGAAACTTACTACTTCACGGAGACGATGATGAGTTCGGGTGTGGTACTCGACCTATCGGAAATACCGGGAACGAAGGTGAAC
F  L  M  A  V  Y  F  R  H  L  D  E  E  E  T  Y  Y  F  T  E  T  M  M  S  S  G  V  V  L  D  L  S  E  I  P  G  T  K  V  N
2521
AAGCACTCAACGGGGGGAGTCGGTGACAAAACAACCCTGGTCGTCGCACCTCTTGTGGCTTCTGCTGGGGTACCTGTTGCCAAGATGTCTGGAAGGGCCCTTGGTCACACAGGAGGAACC
K  H  S  T  G  G  V  G  D  K  T  T  L  V  V  A  P  L  V  A  S  A  G  V  P  V  A  K  M  S  G  R  A  L  G  H  T  G  G  T
2641
ATCGACAAATTGGAGTCGATTCCTGGGTTTCGGACGGAACTTTCCCTTGAA
I  D  K  L  E  S  I  P  G  F  R  T  E  L  S  L  E
```

FIG. 1. Cloning and sequence determination of the region of *T. maritima drrA* and *hpkA* genes. (A) A schematic drawing indicates the location and orientation of *drrA* and *hpkA*. Sequences were determined on both strands for two overlapping restriction fragments, identified by colony hybridization in two minilibraries, represented by hatched rectangles. Numbers refer to base pair positions in the sequence below; R and H indicate the positions of *Eco*RI and *Hin*dIII restriction sites, respectively. (B) The nucleotide sequence of the *drrA-hpkA* region is shown with the deduced amino acids of ORFs encoding DrrA, HpkA, and part of a pyrimidine nucleoside phosphorylase (Pdp) homolog indicated below the DNA sequence. Asterisks mark the translation termination codons in the ORFs. Potential Shine-Dalgarno motifs are underlined.

```
B.s.                                        MNKYRVRLFS VFVVCMILVF CVLGLFLQQL

B.s.   FETSDQRKAE EHIEKEAKYL ASLLDAGNLN NQANEKIIKD AGGALDVSAS VIDTDGKVLY

B.s.   GSNGRSADSQ KVQALVSGHE GILSTTDNKL YYGLSLRSEG EKTGYVLLSA SEKSDGLKGE
E.c.                                   M LERLSWKRLV LELLLCCLPA FIL.GAFFGY
       1
T.m.   MSVFLFVIVA VLFVLLFLVF KKRLSEYKIL IEKLSDMLGE KGVPPLY... .........
B.s.   LWGMLTASLC TAFIVIVYFY SSMTSRYKRS IESATNVATE LSKGNYDART YGGYIRRSDK
E.c.   LPWFLLASVT GLLIWHFWNL .LRLSWWLWV DRSMTPPPGR GS........ .......WEP
       48
T.m.   LFERLKKYVD NLKETISRVE VSRDNFLTIL NSLSEPIFIL DREGKITFLN .EIARELVQG
B.s.   LGHAMNSLAI DLMEMTRTQE MQRDRLLTVI ENIGSGLIMI DGRGFINLVN RSYAKQPHIN
E.c.   LLYGLHQMQL RNKKRRRELG NLIKRFRSGA ESLPDAVVLT TEEGGIFWCN GLAQQILGLR
       107
T.m.   RINPEGRPYY EIFEDYYINE MVEETIKSEE PQEGTLVTYV GNEKKYFHVK VIPVELKSGD
B.s.   PNHMLRRLYH DAFEHEEVIQ LVEDIFMTET KKCKLLRLPI KIERRYFEVD GVPIMGPDDE
E.c.   WPEDNGQNIL NLLRYPEFTQ YL......KT RDFSRPLNLV LNTGRHLEIR VMPY.....T
        167                          H
T.m.   .KIFVILFHD VTKERKLDEM RREFIATVSH ELRTPLTSIH GYAETLLEDD LENKELVKRF
B.s.   WKGIVLVFHD MTETKKLEQM RKDFVANVSH ELKTPITSIK GFTETLLDGA MEDKEALSEF
E.c.   HKQLLMVARD VTQMHQLEGA RRNFFANVSH ELRTPLTVLQ GYLEMMNEQP LEG.AVREKA
       226
T.m.   LKIIEEESAR MTRLINDLLD LEKIEESEAN FEMKDVDLCE VIEYVYRIIQ PIAEENEVDL
B.s.   LSIILKESER LQSLVQDLLD LSKIEQQNFT LSIETFEPAK MLGEIETLLK HKADEKGISL
E.c.   LHTMREQTQR MEGLVKQLLT LSKIEAAPTH LLNEKVDVPM MLRVVEREAQ TLSQKKQ.TF
       286                N                                        G1
T.m.   IVEC..EDVV VRGNKERLIQ MLLNLVDNAV KYTSLKEKGE KKVWVRAYDT PDWVVVEVED
B.s.   HLNVPKDPQY VSGDPYRLKQ VFLNLVNNAL TYTPEGGSVA INVKPREKD. ...IQIEVAD
E.c.   TFEI.DNGLK VSGNEDQLRS AISNLVYNAV NHTPEGT... .HITVRWQRV PHGAEFSVED
       344          F              G2
T.m.   TGPGIPKEAQ SRIFEKFYRV DKARSRKMGG TGLGLTIVKT IVDKHGGKIE VESEINQGTL
B.s.   SGIGIQKEEI PRIFERFYRV DKDRSRNSGG TGLGLAIVKH LIEAHEGKID VTSELGRGTV
E.c.   NGPGIAPEHI PRLTERFYRV DKARSRQTGG SGLGLAIVKH AVNHHESRLN IESTVGKGTR
       404
T.m.   MRVLLPKRR
B.s.   FTVTLKRAAE KSA
E.c.   FSFVIPERLI AKNSD
```

FIG. 2. Amino acid sequence alignment of *T. maritima* (T.m.) HpkA with *E. coli* (E.c.) PhoR (12) and *B. subtilis* (B.s.) PhoR (22). Residues that are strictly conserved in these three proteins are indicated in boldface. Hydrophobic sequences that may form membrane-spanning regions are underlined. Conserved motifs present in the histidine protein kinase family, designated H, N, G1, F, and G2, are indicated by lines above the corresponding sequences. Numbers are indicated for amino acid positions in *T. maritima* HpkA.

tion (13, 28). Whether the high sequence similarity of *T. maritima* HpkA and DrrA to these pairs reflects functional similarity can only be definitively addressed by in vivo studies in *T. maritima.*

Expression and purification of *T. maritima* two-component proteins. For expression of DrrA, the plasmid pJL9B was constructed with the complete *drrA* coding region expressed from

```
                1                                                      50
T.m. DrrA   MAKKKILVVD DDPAILELVG YNLSKEGYEV LKAYDGEEAL KIANDEDVDM
B.s. YycF   .MDKKILVVD DEKPIADILE FNLRKEGYEV HCAHDGNEAV EMVEELQPDL
B.s. PhoP   .MNKKILVVD DEESIVTLLQ YNLERSGYDV ITASDGEEAL KKAETEKPDL
E.c. PhoB   .MARRILVVE DEAPIREMVC FVLEQNGFQP VEAEDYDSAV NQLNEPWPDL

                51                                                     100
T.m. DrrA   FIVDIMLPGI DGFELVRKIR SMEKYKNTPV IFLSAKGEEF DKVLGLELGA
B.s. YycF   ILLDIMLPNK DGVEVCREVR ...KKYDMPI IMLTAKDSEI DKVIGLEIGA
B.s. PhoP   IVLDVMLPKL DGIEVCKQLR ..QQKLMFPI LMLTAKDEEF DKVLGLELGA
E.c. PhoB   ILLDWMLPGG SGIQFIKHLK RESMTRDIPV VMLTARGEEE DRVRGLETGA

                101                                                    146
T.m. DrrA   DDYITKPFSV RELLARVKAI FRR.LSTATQ SK...EERPK KIIAKDLEID
B.s. YycF   DDYVTKPFST RELLARVKAN LRRQLTTAPA EE...EPSSN EIHIGSLVIF
B.s. PhoP   DDYMTKPFSP REVNARVKAI LRRSEIRAPS SEMKNDEMEG QIVIGDLKIL
E.c. PhoB   DDYITKPFSP KELVARIKAV MRRISPMAV. ........EE VIEMQGLSLD

                147                                                    196
T.m. DrrA   VEKYEVKVRG KKVNLTPLEF ELLRFLAENE GKVFSRDVLL DKLWGYDYYG
B.s. YycF   PDAYVVSKRD ETIELTHREF ELLHYLAKHI GQVMTREHLL QTVWGYDYFG
B.s. PhoP   PDHYEAIFKE SQLELTPKEF ELLLYLGRHK GRVLTRDLLL SAVWNYDFAG
E.c. PhoB   PTSHRVMAGE EPLEMGPTEF KLLHFFMTHP ERVYSREQLL NHVWGTNVYV

                197                                                    239
T.m. DrrA   DTRTVDVHIR RLR.TKIEED PSNPKYIITV RGKGYKFRDP GKED
B.s. YycF   DVRTVDVTVR RLR.EKIEDN PSHPNWIVTR RGVGYYLRNP EQD
B.s. PhoP   DTRIVDVHIS HLRPTKIENN TKKPIYIKTI RGLGYKLEEP KMNE
E.c. PhoB   EDRTVDVHIR RLR..KALEP GGHDRMVQTV RGTGYRFSTR F
```

FIG. 3. Amino acid sequence alignment of *T. maritima* (T.m.) DrrA with *E. coli* (E.c.) PhoB (11) and *B. subtilis* (B.s.) PhoP (9, 21) and YycF (14). Residues that are strictly conserved in all four proteins are indicated in boldface, and Asp-54, presumed to be the site of phosphorylation, is underlined. Numbers are indicated for amino acid positions in *T. maritima* DrrA.

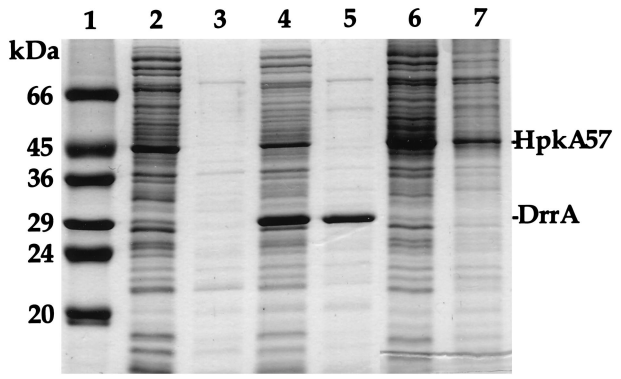

FIG. 4. Expression of *T. maritima drrA* and *hpkA57* in *E. coli* analyzed by SDS-PAGE. Cell lysates (lanes 2, 4, and 6) were compared with heat-treated cell lysates prepared by incubation at 70°C for 10 min followed by centrifugation to remove precipitated proteins (lanes 3, 5, and 7). Lane 1, molecular mass standards; lanes 2 and 3, *E. coli* E507(pJES307); lanes 4 and 5, *E. coli* E507(pJL9B); lanes 6 and 7, *E. coli* E507(pJLR57).

a T7 promoter under control of an IPTG-inducible gene for T7 RNA polymerase. *E. coli* E507 containing pJL9B was analyzed for DrrA expression by SDS-PAGE. Upon IPTG induction, a protein which migrated on SDS-PAGE with approximately the mobility expected for the *T. maritima drrA* product (27.6 kDa) was detected in the soluble fraction of cell lysates from *E. coli* E507(pJL9B) but not from E507 carrying the parent vector pJES307 (Fig. 4, lanes 2 and 4). Partial purification was achieved by heating the soluble fraction of cell lysates at 70°C. The majority of the host proteins precipitated and could be removed by centrifugation (Fig. 4, lanes 3 and 5).

The plasmid pJL26R, a derivative of pJES307, contains the full-length *T. maritima hpkA* gene (see Materials and Methods). No overexpressed protein was visible in cell lysates of *E. coli* E507(pJL26R) upon IPTG induction (data not shown). This was not unexpected since high-level expression of transmembrane proteins in *E. coli* is not commonly achieved. Therefore, we constructed vectors encoding four truncated HpkA proteins each lacking a segment of N-terminal amino acid sequences including the putative transmembrane domain. Plasmids pJLR22, pJLR47, pJLR57, and pJLR65 were constructed for expression of HpkA22, HpkA47, HpkA57, and HpkA65 proteins lacking 22, 47, 57, and 65 N-terminal amino acids, respectively. In all cases, HpkA proteins of approximately the expected sizes were found in the soluble fractions after lysis of cells induced with IPTG (data not shown). HpkA57 was expressed in *E. coli* E507 at a slightly higher level than the other three constructs. Therefore, HpkA57, which migrated with a mobility corresponding to a protein of approximately 45 kDa, was used for subsequent experiments. A similar approach was taken to partially purify HpkA57 by heat denaturation at 70°C followed by centrifugation (Fig. 4, lanes 6 and 7). The success of heat denaturation as an efficient step to obtain partially purified *T. maritima* proteins demonstrates the intrinsic thermostability of the recombinant two-component proteins expressed in *E. coli.*

Phosphorylation activities of recombinant *T. maritima* HpkA and DrrA. Autophosphorylation and phosphotransfer activities of mesophilic two-component proteins can be assayed in vitro with [γ-^{32}P]ATP. Incubation of partially purified *T. maritima* HpkA57 with [γ-^{32}P]ATP in the presence of Mg^{2+} produced a single labeled species that migrated in SDS-PAGE with a mobility corresponding to that of HpkA57 (Fig. 5, lanes 2 and 5). This phosphorylated product was identified as

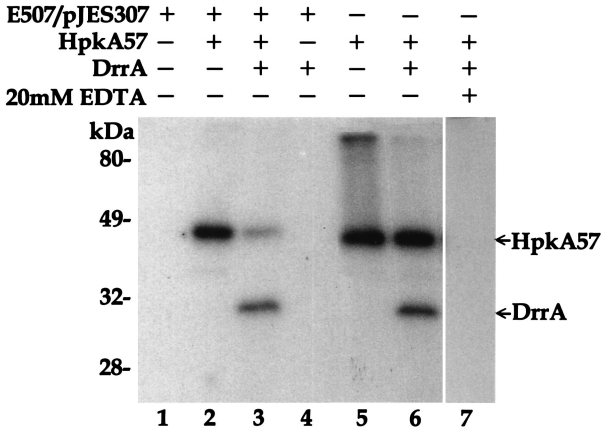

FIG. 5. Phosphorylation of HpkA57 and DrrA. Partially purified *T. maritima* proteins from heat-treated cell lysates were incubated in the presence of [γ-^{32}P]ATP for 10 min at 45°C, separated by SDS-PAGE, and subjected to autoradiography as described in Materials and Methods. The variable components in the reaction mixtures are indicated above each lane. Numbers on the left indicate the molecular sizes of prestained molecular mass standards.

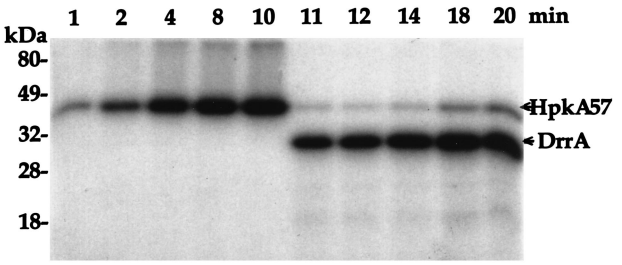

FIG. 6. Autophosphorylation of HpkA and phosphoryl transfer to DrrA. Partially purified HpkA57 (final concentration, approximately 2 pmol/μl) from heat-treated cell lysates was incubated in the presence of [γ-^{32}P]ATP at 45°C as described in Materials and Methods. At the indicated times, 10-μl aliquots were removed and the reactions were quenched. At 10.3 min, partially purified DrrA from heat-treated cell lysates was added to a final concentration of approximately 3 pmol/μl, with less than 5% dilution of the reaction mixture. Subsequently, 10-μl aliquots were removed and the reactions were quenched. An autoradiograph of an SDS gel electrophoretogram is shown. The positions of molecular mass standards are indicated on the left.

HpkA57 since similar extracts prepared from E507 containing the parent vector pJES307 lacked this phosphorylated species (Fig. 5, lane 1). Autophosphorylation was observed with all four partially purified HpkA constructs (data not shown). As is the case for several mesophilic histidine protein kinases, the N-terminal hydrophobic region is not required for kinase activity of *T. maritima* HpkA. Inclusion of EDTA abolished phosphorylation, indicating that phosphorylation requires a divalent cation, such as Mg^{2+} (Fig. 5, lane 7). In preparations without heat treatment, a faintly labeled band was observed migrating at approximately 32 kDa, slightly more slowly than DrrA. This additional band was also observed in unheated cell lysates of E507 with parent vector pJES307 only, suggesting that this phosphorylated species corresponded to a host strain- or vector-encoded protein.

The phosphotransfer activity of *T. maritima* proteins was investigated by incubating [γ-^{32}P]ATP with partially purified HpkA57 and DrrA. In addition to ^{32}P-labeled HpkA57, a second labeled species was observed migrating with a mobility corresponding to DrrA (Fig. 5, lanes 3 and 6). In the absence of HpkA57, no phosphorylation of DrrA was observed (Fig. 5, lane 4). The results demonstrate the transfer of a phosphoryl group between HpkA57 and DrrA and provide biochemical evidence for two-component signaling in *T. maritima*. Phosphoryl transfer was further examined by adding DrrA to the reaction mixture after approaching a steady-state level of HpkA57 phosphorylation by incubation of HpkA57 with [γ-^{32}P]ATP for 10 min (Fig. 6). Upon addition of DrrA, the level of phosphorylation of HpkA57 immediately decreased and DrrA was phosphorylated. Thus, under the conditions of the assay, phosphoryl transfer to DrrA proceeded more rapidly than autophosphorylation of HpkA57. At later times, the level of HpkA57 phosphorylation increased, suggesting that dephosphorylation of phospho-DrrA had become the rate-limiting step of the phosphoryl transfer reaction or, alternatively, providing evidence for back-transfer of phosphoryl groups from phospho-DrrA to HpkA57.

Temperature dependence. Recombinant thermophilic proteins commonly show increased activity with increasing temperatures (1). The autophosphorylation of HpkA57 and the phosphoryl transfer to DrrA were examined by incubating HpkA57 alone (Fig. 7, lanes 1 to 4) or together with DrrA (Fig. 7, lanes 5 to 8) with [γ-^{32}P]ATP for 10 min at temperatures ranging from 4 to 70°C. Increased levels of HpkA57 phosphorylation were observed at the higher temperatures. Similarly, both HpkA57 and DrrA showed increased levels of phosphorylation when incubated with ATP at 37 and 70°C. The level of phosphorylation is influenced by the rates of autophosphorylation and phosphoryl transfer, but also by the intrinsic or catalytically influenced instability of the phosphorylated residues, presumably phosphohistidine in HpkA57 and phosphoaspartate in DrrA. Further analysis is required to determine the specific effects of temperature on each of these reactions.

Conclusions. The presence of *T. maritima* genes encoding histidine protein kinases and response regulators, homologous to those in *E. coli* and other mesophilic bacteria (reference 30 and this study), underscores the universality of the phosphotransfer signal transduction system. The *T. maritima* histidine protein kinases and response regulators are likely to share many features with their mesophilic homologs but may also exhibit certain adaptations that allow function at elevated temperatures. For instance, the aspartyl phosphates of the response regulator proteins are susceptible to hydrolysis and in

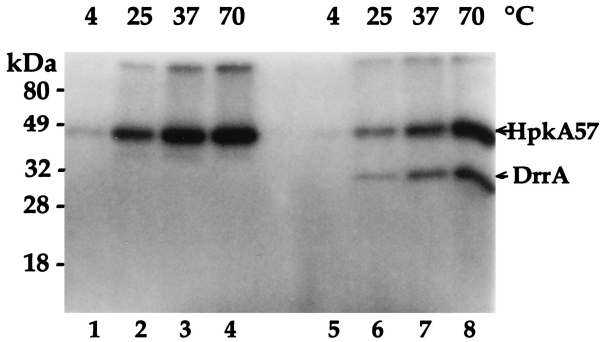

FIG. 7. Temperature dependence of autophosphorylation of HpkA and phosphotransfer between phospho-HpkA and DrrA. Partially purified HpkA57 from heat-treated cell lysates (lanes 1 to 4) or partially purified HpkA57 and DrrA from heat-treated cell lysates (lanes 5 to 8) were incubated in the presence of [γ-^{32}P]ATP for 10 min at the indicated temperatures as described in Materials and Methods. The reactions contained approximately 20 pmol of HpkA57 and 30 pmol of DrrA in a total volume of 10 μl. An autoradiograph of an SDS gel electrophoretogram is shown with numbers at the left indicating the molecular mass of prestained standards.

E. coli proteins have half-lives ranging from seconds to hours at ambient temperatures. It might be expected that significant adaptations of the active site would have to be made to achieve similar half-lives in a protein at 80°C, the optimal growth temperature for *T. maritima*. Intriguing differences such as this, together with the overall similarity that allows the proteins to be used as models for their mesophilic homologs, make these *T. maritima* proteins of interest for further study. The thermostability of these proteins and their expected rigidity at ambient temperatures may also provide advantages for structural analysis.

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