A Two-component System Mediates Developmental Regulation of Biosynthesis of a Heterocyst Polysaccharide*

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Some cyanobacteria couple oxygenic photosynthesis in vegetative cells with O₂-sensitive N₂ fixation in differenti- ated cells called heterocysts. Heterocyst differen- tiation involves extensive biochemical and structural changes that collectively permit heterocysts to assimilate N₂ aerobically and supply the products of N₂ fixation to vegetative cells. HepK and DevR are required for the development of functional heterocysts in Anabaena and Nostoc, respectively. We show that HepK is an autokinase and that Anabaena DevR is its cognate response regulator, together comprising part or all of a two-component system that mediates developmental regulation of biosynthesis of a heterocyst envelope polysaccharide. Recombinant N-hexahistidine-tagged HepK (H₆HepK), the cytoplasmic portion H₆HepK of H₆HepK, and H₆DevR, se overexpressed in Escherichia coli and purified to homogeneity. H₆HepK, but not H₆HepK, autophosphorylates with [γ⁴⁰P]ATP. ADP, specifically, elicits dephosphorylation of phosphorylated H₆HepK. The phosphor- yl group of H₆HepK is transferred rapidly and efficiently to both H₆DevR and H₆DevR, but not to His-tagged OmpR, whose cognate sensor kinase is EnvZ. Sequence comparisons, the results of site-specific mutagenesis, and tests of chemical stability support identification of HepK-His⁴⁴⁸ and DevR-Asp⁵³ as the phosphorylated residues. The muta- tion HepK-I348A abolishes both in vitro autokinase ac- tivity and in vivo functionality of HepK. Heterocysts of both Anabaena and devR Anabaena lack an envelope polysaccharide layer and are nonfunctional. Consistent with the normal site of deposition of that polysaccharide, a hepKgfp transcriptional fusion is expressed principally in proheterocysts. HepK/DevRA is the first two-component system identified that regulates the biosynthesis of a polysaccharide as part of a patterned differentiation process.

When combined nitrogen becomes limiting, Anabaena species and their close relatives respond by initiating a developmental program that results in the production of terminally differentiated, nitrogen-fixing cells called heterocysts. Normally, approximately 10% of the cells become heterocysts that are present singly at semiregular intervals along the filaments, forming a spacing pattern. By sequestering nitrogen within heterocysts, Anabaena spp. can carry out, simultaneously, oxygenic photosynthesis and the O₂-labile assimilation of N₂. Differences between heterocysts and their progenitor vegetative cells enable the heterocysts to provide a microoxic environment for nitrogenase (1). They lack the oxygen-producing reaction of photosystem II; respire rapidly; and have, outside of their cell wall, a layer of glycolipids that impedes the entry of oxygen and an outer, protective layer of polysaccharide (2, 3).

Genes involved specifically in heterocyst differentiation and spacing have been cloned and characterized (4–9). However, no overall regulatory strategy has been identified, and the de- tailed mechanisms that regulate differentiation remain largely unknown. It has been shown, however, that transcription of hepA, whose product is a member of the family of ABC trans- porters and is essential for biosynthesis of heterocyst envelope polysaccharide (4), is controlled by (i) HepK, a putative protein-histidine kinase (10); (ii) HepC, which resembles galactosyl-PP-undecaprenol synthetase (10); and (iii) two unusual site-spe- cific DNA-binding proteins (11).

Polysaccharides, a major class of macromolecules, have great structural diversity. Once thought of only as reserve materials or inert structural elements, they are now recognized as play- ing an important role in the growth and development of plants (12, 13), cell recognition and adhesion (14), hemostasis and lipid metabolism (15, 16), pathogenesis (17), symbiosis (18, 19), and defense responses (20, 21). Different polysaccharides have been linked to carcinogenesis (15, 22) and shown to have anti- cancer activity in vitro, in vivo, and in human clinical trials (23, 24). Far more research on polysaccharides has emphasized their structure and biosynthesis than the regulation of their biosynthesis. Developing heterocysts provide a model of patterned, functional differentiation in which synthesis of a specific polysaccharide is an essential feature. The structure of heterocyst envelope polysaccharide has been determined for several species (25, 26), the broad outlines of its biosynthesis have been examined (27), and a cluster of genes has been presumptively identified (28, 29) that is involved in its biosyn- thesis. However, the regulation of its synthesis remains largely uncharted.

Two-component phosphorelay regulatory systems are the principal means for coordinating responses to environmental changes in bacteria and are found also in plants, fungi, protozoa, and amoebae (30–32). Typically, such a system consists of a membrane-associated sensor kinase and its cognate response regulator. Most known sensor kinases consist of a cytoplasmic N terminus, two transmembrane segments linked by a periplasmic bridge, and a cytoplasmic, C-terminal, transmitter portion with a conserved histidine residue. Signal reception by the kinase stimulates an ATP-dependent autophosphorylation of that histidine. Transfer of the phosphate group to a con-
served aspartyl residue of the response regulator renders the latter functional. Most known response regulators function as transcription factors whose effector domains bind DNA and regulate the expression of certain genes, effecting an adaptive response (33). However, some response regulators lack a known effector domain (32), and some effector domains are enzymatically active (34, 35). When signaling subsides, both components undergo dephosphorylation, inactivating the response regulator.

Genomic sequence data have identified no presumptive vertebrate, nematode, or fruit fly genes that encode two-component phosphorelay proteins (36–38). Because some two-component phosphorelay proteins are essential for the virulence of microbial pathogens including human fungal and bacterial pathogens, novel anti-microbial drugs targeted to two-component phosphorelay systems may prove highly specific for microbial pathogens. Although more than 1000 two-component phosphorelay proteins have been predicted from genomic data, very few have an identified function and have been shown experimentally to participate in phosphorelay processes. Some predicted homologs of histidine kinases may not be kinases or may not phosphorylate a histidine residue (32). For example, Azotobacter vinelandii NifL contains five conserved blocks of amino acids characteristic of histidine sensor kinases (39) but has not been observed to autophosphorylate (40, 41), and DivL (42), pyruvate dehydrogenase kinase (43), and plant phytochromes (44) phosphorylate a tyrosine or serine rather than a histidine.

Predicted proteins HepK from Anabaena sp. strain PCC 7120 and DevR from Nostoc punctiforme strain ATCC 29133 resemble respectively a sensory protein-histidine kinase (10) and a response regulator that lacks known DNA-binding domains (45). The amino acid sequence of Anabaena sp. ortholog DevR α of DevR but for a possible 30-amino acid, N-terminal extension, is 93% identical and 98% similar to that of Nostoc DevR. Unlike most response regulators, DevR and DevR have a 7-amino acid peptide, SRSVQYGSG (46), in the γ-turn loop region. DevR, but not DevR, from which that peptide was deleted, is weakly phosphorylated in vitro by Escherichia coli sensor kinase EmfZ, a result that supported the interpretation of Hagen and Meeks (46) that DevR is a response regulator in an unidentified phosphorelay system that controls heterocyst maturation in N. punctiforme strain ATCC 29133. However, no cognate histidine kinase was identified for DevR. No gene that evidently encodes a response regulator neighbors hepK. devR α is 1.4 Mb distant from hepK (28). hepK Anabaena sp. and devR N. punctiforme cannot assimilate N₂ aerobically (10, 45). We present evidence that Anabaena sp. HepK is a histidine kinase, that DevR α is its cognate response regulator, and that the two comprise part or all of a two-component system that controls the biosynthesis of heterocyst envelope polysaccharide.

EXPERIMENTAL PROCEDURES

Culture and Preparation for Electron Microscopy—Anabaena sp. strain PCC 7120 and derivatives of it were grown at 30 °C in the light at approximately 140 microeinstein m⁻² s⁻¹ (Li-Cor Quantum Radiometer/Photometer model LI-185A; Lincoln, NE) on AA + N agar (47) in the presence of appropriate antibiotics or on a rotary shaker in medium AA/S or AA + N (47) plus appropriate antibiotics. Wild-type and devR α, Anabaena sp. strains and wild-type N. punctiforme strain ATCC 29133 and its dev R derivative UCD311 were prepared for electron microscopy (48) after the following regimens of growth. Anabaena sp. strains grown 5 days in AA/S + N (plus, for the mutant strain, 2 µg of spectinomycin sulfate ml⁻¹) were sedimented, washed three times with AA/S, and incubated for 3 days on a Nuclepore Rec-85 membrane at 4 °C. Wild-type N. punctiforme was grown for 5 days in AA/S. UCD311 was incubated 2 days in AA/S after 7 days of growth in AA/S + N plus 10 µg of neomycin sulfate ml⁻¹ and three washes with AA/S. DNA Manipulation—Recombinant DNA procedures were performed in a standard manner (49). The enzymes were purchased from New England Biolabs, Invitrogen, and Roche Applied Science and used as recommended by the manufacturer.

Construction of Expression Plasmids—Truncated hepK (‘hepK’) encoding residues 267–575 of HepK that lack the two presumptive transmembrane regions and full-length hepK (GenBank accession number U68034) were amplified by PCR with, respectively, primer pairs 5′-GG-GATCTCATATGCGGACTGGAGGATCTGACAG-3′ and 5′-GAAGACTCAGGATCTTGGCAGCAGGACCGC-3′ (CPW99) or 5′-GGATCTCATATGCGGACTGGAGGATCTGACAG-3′ and 5′-GAAGACTCAGGATCTTGGCAGCAGGACCGC-3′ (CPW99) (introduced Ndel and BglI sites are underlined) with pRL2079 (10) as template. The PCR products were cloned into the Ndel and BamHI sites of plasmid pET-14b (Novagen, Inc., Madison, WI), which provided an N-terminal hexahistidine tag. To reduce the extent of PCR product degradation, primers were designed to be flanked by the regions in intact hepK was replaced by a portion of wild-type hepK was as a smaller portion in truncated hepK. The resulting plasmids were denoted pRL2406 and pRL2433, respectively. devR and devR24 (encoding DevR37 of N. punctiforme) were PCR-amplified from plasmids pSRC169 and pSRC345 (Ref. 46; gifts of J. C. Meeks), respectively, as templates with the primers 5′-GGAAATCCATATGCAAACATGTGTCG-3′ and 5′-GGAAATCCATATGCAAACATGTGTCG-3′, were cloned between the Ndel and BamHI sites of pET-14b to produce pRL2749 for production of H6DevR. The ompR gene (GenBank accession number J06156) of E. coli strain MC1060 in plasmid pLAN801 (a gift of Dr. M. Igo) was amplified by PCR with the primers 5′-GGAAATCCATATGCAAACATGTGTCG-3′ and 5′-GGAAATCCATATGCAAACATGTGTCG-3′, was cloned between the Ndel and BamHI sites of pET-14b to produce pRL2146 and pRL2487. pSRC351, encoding H6DevR-D53Q, was also cloned from Dr. Meeks. devR α, PCR-amplified from total Anabaena sp. DNA with the primers 5′-GGAAATCCATATGCAAACATGTGTCG-3′ and 5′-GGAAATCCATATGCAAACATGTGTCG-3′, was cloned between the Ndel and BamHI sites of pET-14b to produce pRL2749 for production of H6DevR. The ompR gene (GenBank accession number J06156) of E. coli strain MC1060 in plasmid pLAN801 (a gift of Dr. M. Igo) was amplified by PCR with the primers 5′-GGAAATCCATATGCAAACATGTGTCG-3′ and 5′-GGAAATCCATATGCAAACATGTGTCG-3′, was cloned between the Ndel and BamHI sites of pET-14b, yielding pRL2484. All of the above PCR products were proven error-free by DNA sequencing.

Site-directed Mutations of hepK — Anabaena sp. strain PCC 7120 medium was used for PCR mutagenesis (QuickChange site-directed mutagenesis; Stratagene) was used to change the conserved His348 to Ala at the presumptive site of phosphorylation of HepK to produce a mutant truncated HepK protein, which we denote HepK-H348A. The mutagenic oligonucleotides 5′-ATTCGATCGATCGATCGATCGATCG-3′ and 5′-ATTCGATCGATCGATCGATCGATCG-3′, was cloned between the Ndel and BamHI sites of pET-14b to produce H6DevR-D53Q, H6DevR, H6OmpR, and H6EnvZc, which were PCR-amplified from plasmids pRL2406 as template were used for PCR; the product was cloned in pET-14b, producing pRL2454. The sequence of the 310-bp BstEII-BstX1 fragment of pRL2454 was shown to be identical to the corresponding fragment of pRL2406, except for the H348A substitution, by DNA sequencing. Transfer of the BstEII-BstX1 fragment from pRL2406 to pRL2406 produced pRL2446, in which H6HepK-H348A was produced and purified as had been H6 HepK.

Test of Complementation of hepK Mutant Y7 with hepK H348A—Transposon-generated hepK mutant Y7 cannot grow aerobically on N₂, but Y7 (pRL2078) can (10). pRL2406, identical to pRL2078 but for an H348A change in hepK, was constructed by transferring the 4.9-kb hepK-bearing HB112 DNA fragment from pRL2078 to pRL2406, which lacks BstEII and BstX1 sites; replacing the 310-bp BstEII-BstX1 fragment of pRL2454 to produce Y7 (pRL2406) produced pRL2406 in which H6HepK-H348A was produced and purified as had been H6 HepK.

Production and Purification of Heterocyst-tail-Derived Peptides—To obtain the His-coded peptides H2 HepK, H2 DevR, H2 OmpR, H2 EnvZc, H2 DevR7, HepK-D53Q, HepK-D53Q, H2 OmpR, and H2 EnvZc, E. coli strain BL21 (DE3) was transformed with pRL2406, pRL2433, pRL2457, pRL2479, pRL2461, pRL2487, pSRC351, pRL2484, and pPH001 (a gift of M. Inouye) (52), respectively. Each transformant was cultured in 500 ml of LB medium supplemented with 100 µg of ampicillin ml⁻¹ at 37 °C to an absorption of 0.6–0.7 at 600 nm isopropyl-β-D-thiogalactopyranoside was added, and incubation was continued for 3 h. The His-coded tagged proteins were purified to homogeneity as follows. E. coli suspended in 50 mM sodium phosphate, 100 mM NaCl, pH 7.0, was broken with a French press (American Instrument Co. Div. Travellon Laboratories, Inc., Silver Spring, MD) and centrifuged. The supernatant was dialyzed overnight against 50 mM sodium phosphate, 150 mM NaCl, pH 7.5, and applied to a Ni-nitrilotriacetic acid (Neurobio technologies, Inc., Huntsville, AL) column. The column was extensively washed, and the bound proteins were eluted with a linear gradient of 50–550 mM imidazole in 50 mM sodium phosphate, 150 mM NaCl, pH 7.5. All subsequent steps were run at 4 °C.

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Spring, MD). The supernatant solution from centrifugation for 20 min at 200,000 × g at 4 °C was applied to a cobalt-based resin column (Clontech), which was then washed thrice with 50 mM sodium phosphate, 300 mM NaCl, 5 mM imidazole, pH 7.0. The intended protein, eluted with 50 mM sodium phosphate, 300 mM NaCl, pH 5.5, was loaded onto a Sephadex G-100 gel filtration column (Amersham Biosciences) from which it was eluted at 4 °C with 50 mM sodium phosphate, 100 mM NaCl, pH 7.0, at 20 ml h⁻¹.

Assays of Phosphorylation and Transphosphorylation—Unless otherwise specified, phosphorylation reaction mixtures contained 25 mM Tris-HCl, pH 8.0, 50 mM NaCl, 5 mM MgCl₂, 1 mM dithiothreitol, 0.5 mM EDTA, and 30–80 μg [γ⁻³²P]ATP (6,000 Ci mmol⁻¹). H6HepK, H6DevR, or derivatives of them, H6DevRα, H6EnvZ, and H6OmpR were added to final concentrations of 2.5 μM or as indicated. The reactions were initiated by the addition of the radioactive substrate, incubated at room temperature (approximately 24 °C) for different times, and stopped by the addition of an equal volume of 2× concentrated SDS-PAGE loading buffer (50 mM Tris-HCl, pH 6.8, 4% SDS, 10% glycerol, 200 mM dithiothreitol, 0.003% bromphenol blue). The reactions, once stopped, were immediately subjected to SDS-PAGE. After completion of electrophoresis, the gels were electrobotted onto polyvinylidine difluoride membranes (Bio-Rad) at 200 mA in protein-transfer buffer (25 mM Tris, 250 mM glycine, 5% methanol, 0.1% SDS, pH 8.3) at 4 °C for 1 h, and the membrane was then air-dried at room temperature. The radioactivity of proteins attached to polyvinylidine difluoride membranes was determined qualitatively by autoradiography with Hyperfilm (Amersham Biosciences) or quantitatively with a PhosphorImager model V5.6 (Molecular Dynamics, Sunnyvale, CA). To show the protein bands, the polyvinylidine difluoride membrane was later stained with Coomassie Brilliant Blue R-250 for 2 min and then destained with 50% methanol.

In time course experiments, portions of a sample were removed, mixed with loading buffer, and kept on ice until all of the samples were taken. To compare transfer of [³²P] from H6HepK-²⁵P to H6DevR and to H6DevRα, one 13-μl fraction and two 40-μl fractions were removed from a solution of 9 μg of phosphorylated H6HepK in 100 μl of standard phosphorylation buffer. Six μg of H6DevR or H6DevRα in 12 μl of standard phosphorylation buffer were added to separate 40-μl fractions of the samples then incubated at 4 °C. At the indicated reaction times, 15-μl portions of samples were rapidly transferred to an equal volume of 2× concentrated SDS-PAGE loading buffer. The mixtures were kept on ice until all of the portions had been removed and were then subjected to SDS-PAGE.

Stability of Phosphate Linkages in Phosphorylated H6HepK, H6DevRα, and H6DevR, and Effects of Nucleoside Diphosphates on Dephosphorylation of H6HepK-²⁵P—To examine the chemical stability of their phosphate linkages, 30 pmol of H6HepK or 50 pmol of H6DevRα or H6DevR with 20 pmol of H6HepK were phosphorylated for 30 min in 30 μl of reaction mixture. The reactions were terminated by adding an equal volume of 2× concentrated SDS-PAGE loading buffer. Samples of 10 μl were pipetted into microcentrifuge tubes containing 1 g of HCl, 0.15 μl of H₂O, and 300 μl of NaOH, giving final pH values of 0.9, 2.7, 7.0, or 12.5, respectively. pH values were confirmed with Color pHast® indicator strips (EM Science, Darmstadt, Germany). The mixtures were incubated for 60 min in a water bath at 42 °C. Another sample, to which 2 μl of water was added, was kept on ice. The samples were separated by SDS-PAGE and analyzed for radioactivity.

The kinetics of loss of [³²P] from H6HepK-²⁵P and H6DevR-²⁵P at pH 12.5 were assessed as follows. H6HepK (35 pmol) was phosphorylated for 30 min in a 30-μl reaction mixture; in some experiments the 35 pmol of H6HepK-²⁵P in 20 μl was then used to phosphorylate 35 pmol of H6DevR for 30 min in a 30-μl reaction mixture, and the protein or protein-diphosphate conjugate was then subjected to SDS-PAGE by the addition of 2× concentrated loading buffer (pH 6.8). Each resulting 60-μl mixture, prewarmed for 2 min at 42 °C, was adjusted to pH 12.5 by addition of 12 μl of 3 N NaOH. pH values were confirmed with Color pHast® indicator strips. At intervals thereafter (see Fig. 6), an 8-μl sample was withdrawn and introduced into a microcentrifuge tube containing 1.33 μl of 3 N NaOH and 2 μl of pHe 7.0. The samples were incubated on ice until sampling was completed. All of the samples were separated by SDS-PAGE and analyzed for radioactivity.

ADP, CDP, GDP, or TDP (sodium salts; Sigma; sodium ADP was also purchased from TCI America for confirmation) at a final concentration of 0.5 mM was incubated for 5 min in a 15-μl phosphorylation reaction mixture containing 2 μg of H6HepK-²⁵P. The reactions were stopped by the addition of 2× concentrated SDS-PAGE loading buffer. The samples subjected to SDS-PAGE were analyzed for radioactivity.

Inactivation of devRA—A 1415-bp DNA fragment containing devRA and DNA downstream from it was amplified by PCR from genomic DNA.
of OmpR, a response regulator whose cognate histidine kinase is EnvZ (54). Derivative H6EnvZc, which lacks the transmembrane regions of EnvZ, autophosphorylated (Fig. 4B, lane 2) and then served efficiently as $^{32}$P donor to H6OmpR (Fig. 4B, lane 3), whereas transfer of $^{32}$P from H6HepK to H6OmpR was not observed (Fig. 4, A and C, lanes 2 and 3).

Previously autophosphorylated H6HepK was mixed with H6DevR and H6DevRA7; 85% of $^{32}$P from H6HepK-$^{32}$P was transferred to H6DevR within 30 s, 97% within 5 min, and an even greater percentage was transferred upon incubation for 1 h (Fig. 5B, lanes 1–3). H6DevRA7 was phosphorylated at least 10-fold more slowly, with approximately 30% of the $^{32}$P transferred by 1 h (Fig. 5B, lanes 5–7; PhosphorImager quantitation not shown). H6DevRA7 alone was not detectably phosphorylated when incubated with $^{32}$P-ATP for 1 h (data not shown).

Stability of Phosphate Linkages to H6HepK, H6DevR, and H6DevR—The chemical stability of the phosphoryl bonds in phospho-H6HepK, -H6DevRA, and -H6DevR was tested. Phosphorylated amino acid residues in proteins are commonly classified into O-phosphates, phosphomonomesters formed by phos-
Plasmid pRL2480 is identical to pRL2078 except that it encodes a gene sequence in the absence of the hexahistidine tag. Unlike H₆HepK (Fig. 8, A and B, lane 1), H₆HepK-D53Q (Fig. 8B, lanes 3–7) is not phosphorylated with [γ-³²P]ATP (the radioactivity here detected was not repeatable observable). Lanes 3, H₆HepK-H348A + [γ-³²P]ATP; lanes 4, H₆HepK-H348A + [α-³²P]ATP; lanes 1 and 2, H₆HepK + [γ-³²P]ATP and + [α-³²P]ATP incubated for 30 min. Lane 1, 1 μg of H₆HepK + [γ-³²P]ATP incubated for 30 min. Lane 3, 1 μg of H₆HepK + [γ-³²P]ATP + 2 μg of H₆DevR incubated for 30 min. A and C, samples were assayed for autophosphorylation by SDS-PAGE followed by autoradiography. B, the same membrane as A stained with Coomassie Brilliant Blue R-250.

Two-component System Regulates Polysaccharide Biosynthesis

DevR and of DevR₄ are phosphorylated. This inference was tested by use of H₆HepK-D53Q (46) and construction and use of H₆HepK-H348A (the numbers 348 and 53 refer to the truncated amino acid sequences in the absence of the hexahistidine tag). Unlike H₆HepK (Fig. 9, A and B, lane 1), H₆HepK-H348A overexpressed in E. coli and purified to homogeneity (Fig. 8A, lanes 3 and 4) failed to autophosphorylate (Fig. 8A, lane 3). H₆DevR-D53Q, similarly overexpressed and purified to homogeneity (data not shown), unlike H₆DevR, did not accept ³²P (Fig. 9C, lanes 2 and 3). hepK mutant Y7 of Anabaena sp. is neomycin-resistant and unable to assimilate N₂ aerobically (10) and is spectinomycin-sensitive and therefore unable to grow in the presence of neomycin, spectinomycin, and fixed nitrogen (Fig. 9C, circle). Plasmid pRL2480, which bears wild-type hepK (10) and confers resistance to spectinomycin, complemented Y7, as shown by the dark green growth of Y7 (pRL2078) (Fig. 9D, circle) in the absence of fixed nitrogen. Plasmid pRL2480 is identical to pRL2078 except that it encoded a new gene sequence in the absence of the hexahistidine tag. Unlike H₆HepK (Fig. 8A, lane 1), H₆HepK-D53Q (Fig. 8B, lanes 3–7) is not phosphorylated with [γ-³²P]ATP (the radioactivity here detected was not repeatable observable). Lanes 3, H₆HepK-H348A + [γ-³²P]ATP; lanes 4, H₆HepK-H348A + [α-³²P]ATP; lanes 1 and 2, H₆HepK + [γ-³²P]ATP and + [α-³²P]ATP incubated for 30 min. Lane 1, 1 μg of H₆HepK + [γ-³²P]ATP incubated for 30 min. Lane 3, 1 μg of H₆HepK + [γ-³²P]ATP + 2 μg of H₆DevR incubated for 30 min. A and C, samples were assayed for autophosphorylation by SDS-PAGE followed by autoradiography. B, the same membrane as A stained with Coomassie Brilliant Blue R-250.

ADP elicits the dephosphorylation of H₆HepK-³²P but has no evident effect on H₆HepK-mediated transfer of ³²P to H₆DevR. A, residual radioactivity of prephosphorylated H₆HepK incubated alone (lane 1) or with 0.5 mM ADP, CDP, GDP, or TDP, respectively, for 5 min at room temperature. B, H₆HepK prephosphorylated with [γ-³²P]ATP and incubated for 30 min (lane 3) without further supplement or with the addition of 1 μg of H₆DevR (lane 2), 0.5 mM ADP (lane 4), or 1 μg of H₆DevR plus 0.5 mM ADP (lane 1).
codes a HepK-H348A variant of HepK. In contrast to pRL2078, pRL2480 failed to complement Y7, as shown by the lack of growth in the absence of fixed nitrogen (Fig. 9D) of 10 randomly chosen derivatives of Y7 that bear pRL2480 and are therefore able to grow in the presence of fixed nitrogen, neomycin, and spectinomycin (Fig. 9C).

ADP Elicits Dephosphorylation of HepK-32P but Has No Evident Effect on Phosphotransfer from HepK-32P to DevR—Because autophosphorylation of HepK presumably produces ADP, we tested whether ADP affects autophosphorylation of, or phosphate transfer from, HepK-32P. Unlike incubation with CDP, GDP, or TDP (Fig. 10A, lanes 1 and 3–5), incubation of a solution of phosphorylated HepK for 5 min with 0.5 mM ADP resulted consistently in a loss of ~90% of the 32P from HepK-32P (Fig. 10A, lanes 1 and 2). The loss was even greater after 0.5 h (Fig. 10B, lanes 3 and 4), whereas ADP negligibly affected net phosphate transfer from HepK-32P to DevR (Fig. 10B, lanes 1 and 2). In the absence of HepK

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**Fig. 11. Ultrastructural aspects of devR and devRA mutants.** Heterocysts of wild-type Anabaena sp. (A and B) and wild-type N. punctiforme (E and F) have but those of devR Anabaena sp. derivative DR2481a (C and D) and devRA N. punctiforme mutant UCD311 (G and I) lack, a layer of polysaccharide that envelops the laminated layer of glycolipids (laminae seen in details B, D, F, and I of rectangles within micrographs A, C, E, and G, respectively). H, heterocyst; V, vegetative cell; GL, glycolipid layer; PS, polysaccharide layer.
Figure 11. Inactivation of devR, hepK, or hepK blocks formation of the heterocyst envelope polysaccharide layer—Heterocysts of hepK mutant Y7 of Anabaena sp. form a laminated layer of glycolipids but no polysaccharide layer and fail to fix N₂ aerobically (10). Inactivation of devR by double reciprocal recombination with plasmid pRL2481a (see “Materials and Methods”) was confirmed by diagnostic PCR (data not shown). devR, Anabaena sp. also cannot grow aerobically on N₂. As in hepK Anabaena sp. (10), heterocyst envelopes of devR, Anabaena sp. (Fig. 11, C and D) and devR N. punctiforme (Fig. 11, G and I) retain a laminated glycolipid layer but lack a polysaccharide layer (Fig. 11, compare A, B, E, and F, respectively).

**In Vivo Localization of Expression of hepK—Plasmid pRL2437 bears a hepK::gfp transcriptional fusion (gfp encodes green fluorescent protein) (57).** Fluorescence from this transcriptional fusion was observed primarily in single heterocysts (Fig. 12D, arrow P) but sometimes in paired cells (Fig. 12, B and C, arrow P), of which at least one was a heterocyst. Slight fluorescence of questionable significance in this highly autofluorescent organism, originated from vegetative cells (Fig. 12, A–D) and mature heterocysts (Fig. 12B, arrow H).

**DISCUSSION**

HepK resembles protein-histidine kinases of two-component regulatory systems (10), and DevR resembles certain response regulators (45). On the basis of the following observations, we conclude that HepK is a histidine kinase and that it and DevR function in Anabaena sp. strain PCC 7120 as interacting components of a two-component regulatory system. First, H₆HepK autophosphorylates (Figs. 2–8 and 10) and serves as a phosphodonor, rapidly and efficiently, to H₆DevR (Fig. 3, A and B) and H₆DevR (its N. punctiforme ortholog; Fig. 3, C and D) but (unlike H₆EnvZC) not to H₆OmpR (Fig. 4A). Second, replacement of a conserved amino acid His³⁴⁸ of HepK by Ala prevents both in vitro phosphorylation (Fig. 8) and in vivo function (Fig. 9), and replacement of conserved Asp⁵³ of H₆DevR by Glu prevents its activity as a phospho-acceptor from H₆HepK (Fig. 8) and its in vivo function in N. punctiforme (46). Third, like inactivation of hepK (10), inactivation of devR, or devR blocks formation of a heterocyst envelope polysaccharide layer, while abundant synthesis of the laminated structure composed of glycolipids continues (Fig. 11). Because DevR, the phenotype of a devR mutant, and the N. punctiforme HepK ortholog are highly similar to DevRA, the phenotype of devRA Anabaena, and HepK, respectively, it seems highly likely that DevR and the N. punctiforme HepK ortholog function and interact similarly to DevRA and HepK. We know of no other two-component system that regulates the biosynthesis of a polysaccharide as part of a patterned differentiation process.

Protein-histidine kinases VirA, ArcB, and EnvZ autophosphorylate in vitro when truncated by removal of transmembrane regions but not when intact (54, 55, 58). Similarly, we observed phosphorylation in vitro by truncated but not by intact H₆HepK (Fig. 2). Perhaps intact HepK cannot fold properly in vitro; when overexpressed in *E. coli*, H₆HepK formed mostly inclusion bodies, whereas H₆HepK did not. Alternatively, intact HepK may autophosphorylate only in response to a signal specific to heterocyst development that is lacking in vitro. Sequence alignments (10, 45) suggested that His³⁴⁸ of HepK and Asp⁵³ of DevR and DevR may be phosphorylated. Concordantly, linkage of ³²P to H₆HepK is unstable at pH 2.7 and stable at pH 12.5 (Figs. 6 and 7), behavior typical of phosphorylated histidine or lysine residues, whereas H₆DevRA⁻³²P and H₆DevR⁻³²P are moderately stable at pH 2.7 and unstable at pH 12.5 (Figs. 6 and 7 and data not shown), as is characteristic of acyl phosphates (55, 56). Dephosphorylation of both histidine N-³²P (as observed for H₆HepK⁻³²P; Fig. 6A, lane 2) and aspartate acyl-³²P (as observed for H₆DevR⁻³²P; Fig. 6B, lane 2) at pH 0.9 is expected (55).

Response regulators such as DevRA and DevR that lack a known C-terminal effector domain may (i) interact with a downstream target only in response to phosphorylation (50) or (ii) act as an intermediate in a multicomponent phosphorelay system (33, 60). Both DevR-D53Q (glutamine is unchanged and cannot be phosphorylated) and DevR-D53E (glutamate may structurally mimic phosphorylated aspartate) lack DevR function in vivo in N. punctiforme (46), suggesting that the function of DevR may depend on a role of DevR-Asp⁵³ as a phosphorytransferase intermediate. That is, DevR, like Spo0F, may phosphorylate a hitherto unidentified DNA-binding protein (46).

The genome of Anabaena sp. strain PCC 7120 contains 73 genes for putative simple His kinases, 53 more for putative hybrid His kinases, and 77 genes for putative simple response regulators, in addition to other kinds of protein kinases (61). Few of these have a known function, and interacting pairs have not been demonstrated. Specificity of interaction of a particular regulator with a particular sensor in response to a given environmental stimulus may often be essential to avoid inappropriate responses, although certain response regulators may have to receive input signals from more than one sensor. Although there is very weak cross-talk in vitro between the non-cognate histidine kinase EnvZ, whose cognate response regulator is OmpR (54), and H₆DevR, no cross-talk was observed with H₆DevRΔT (46). Whereas the 7-amino acid peptide, SRSVYQG, located in DevR and DevRA is absent from most known response regulators, a similar insertion is present in FurA, a putative response regulator in *Mycococcus xanthus* (62). Our biochemical data (Fig. 5) showed that deletion of that peptide from the γ-turn loop region of H₆DevR greatly decreased the rate and extent of phosphotransfer from H₆HepK to H₆DevRΔT in vitro and so may influence the rate, and perhaps the specificity, of phosphotransfer from HepK in vivo. The lack of cross-talk between HepK and OmpR (Fig. 4A) confirmed the specificity of recognition between HepK and DevR.

Although the presence of ADP results in dephosphorylation...
of H$_3$HepK$^{32}$P (Fig. 10), we saw no diminution of H$_3$HepK-dependent phosphorylation of H$_3$DevR (Fig. 10B, lanes 1 and 2), perhaps because the latter reaction takes place much more rapidly. On the basis of the observed effect of ADP in vitro, we conjecture that the ratio of ATP to ADP in vivo may regulate the level of phosphorylated H$_3$HepK.

Because a chromosomal hepK:gfp fusion was expressed too weakly for us to realize the resulting fluorescence, we expressed hepK:gfp in a pDU1-based plasmid. Unlike expression of a glnA fusion in a pDU1-based plasmid, which was localized in all cells of N$_2$-fixing filaments (63) as expected on the basis of a fusion in a pDU1-based plasmid, which was localized in all cells of N$_2$-fixing filaments (63) as expected on the basis of a

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