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HepK, a protein-histidine kinase from the cyanobacterium *Anabaena* sp. strain PCC 7120, binds sequence-specifically to DNA

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Abstract

Two-component phosphorelay systems are minimally consisted of a sensory protein-histidine kinase (HK) and a response regulator (RR). HK autophosphorylates its conserved histidine residue in response to stimulus from an environment, this phosphate group then is transferred to a conserved aspartic acid residue of an RR, which is generally a transcription factor. HepK is a member of the family of sensory protein-histidine kinases in two-component phosphorelay systems (TCPS). We previously showed that HepK is an autokinase, and that DevR is its cognate RR, together comprising a mini two-component phosphorelay system that mediates developmental regulation of biosynthesis of a heterocyst envelope polysaccharide in the cyanobacterium *Anabaena* sp. PCC 7120. Unlike a typical TCPS, both HepK and DevR lack known DNA-binding domains. However, mutations in *hepK*, *hepC* and *hepA* all block the synthesis of heterocyst envelope polysaccharide. A *hepK* mutation of *Anabaena* blocks the induction of *hepA* expression. We hypothesized that HepK may regulate transcription of *hepA or hepC* by binding to DNA. To test this hypothesis we have performed a gel-shift analysis and have shown that although lacking a known DNA-binding motif, a truncated, soluble version of HepK binds sequence-specifically to a fragment of DNA found upstream from *hepC*, a gene that is located immediately upstream from *hepA* and also required for the synthesis of heterocyst envelope polysaccharide. The conserved phosphorylation histidine residue of HepK kinase is not required for this DNA-binding activity. Therefore, regulation of the synthesis of heterocyst envelope polysaccharide by HepK may be, at least in part, independent of two-component phosphorylation. The membrane-anchored HepK kinase with specific DNA-binding activity may serve as a membrane-tethered transcription factor, which may require an activation of regulated intramembrane proteolysis. We have found no other example of a protein histidine kinase without a known DNA binding motif that binds DNA sequence-specifically. Our finding may enable development of small DNA molecule as highly specific anti-microbial drugs because protein histidine kinases are broadly conserved in microbial pathogens but absent in humans.

Keywords: Cyanobacterium, heterocyst, *hepA*, *hepC*, histidine kinase binds DNA, DNA drug

Introduction

Two-component phosphorelay regulatory systems are widespread in prokaryotes, and are found also in fungi, amoebae and plants [1,2]. Typically, a sensory protein-histidine kinase phosphorylates its own conserved histidine residue in response to an environmental stimulus. Histidine kinases are not commonly known to function by binding to DNA, although they have been shown to regulate the transcription of target genes indirectly by activation of a specific response regulator. Response regulators generally contain an N-terminal regulatory domain with a conserved aspartate residue, to which the phosphoryl group of the kinase is transferred, and a variable C-terminal effector domain [3]. The response-regulator protein usually regulates the expression of certain genes to effect an adaptive response [4]. Most known

response regulators are transcription factors whose effector domains bind DNA. However, some response regulators have no known effector domain [1] and the effector domain of some others are enzymatically active [5,6].

Three proteins with similarity to members of two-component regulatory systems are known to influence heterocyst development in filamentous cyanobacteria. HepK was identified as a sensory protein-histidine kinase [7] and DevR and PatA resemble response regulators. All three lack known DNA-binding domains [7,9,10]. Mutations in *hepK, hepC* and *hepA* all block the synthesis of heterocyst envelope polysaccharide, rendering the heterocysts incapable of N_2 fixation under aerobic conditions [10,11]. A *hepK* mutation of *Anabaena* sp. strain PCC 7120 blocks the induction of *hepA*, whereas inactivation of *hepC*, located directly upstream from *hepA*, leads to constitutive expression of *hepA* [10].

The catalytic core of histidine kinases, including of HepK, contain highly conserved regions (the H, N, D/F, G1 and G2 boxes) that are distinct from the previously characterized Ser/Thr/Tyr kinase domain [12]. That catalytic core does not appear to be obligatorily dependent upon catalytic phosphorylation of a His rather than a Ser, Thr, or Tyr, because histidine kinase homologs are known in which the phosphorylated His is substituted by Tyr [13] or Ser [14]. Unanticipated similarities of the catalytic core to DNA gyrase B and to DNA-mismatch-repair enzyme MutL [12,15,16] provide hints that a histidine kinase might be able to interact directly with DNA. We previously demonstrated that HepK is a sensory protein-histidine kinase in HepK-DevR two-component regulatory system [7]. Because HepK may regulate the transcription of *hepA* or *hepC*, we tested whether HepK can bind DNA upstream from these genes. We report here that a water-soluble version of HepK that lacks the two putative transmembrane regions of that protein is a histidine kinase that binds sequence-specifically to DNA upstream from *hepC*. Therefore, the regulation of the synthesis of heterocyst envelope polysaccharide by HepK may be, at least in part, independent of two-component phosphorylation. Except for a kinase that bears a presumptive response regulator with a DNA-binding motif [17], we have found no other known example of a histidine kinase that binds DNA sequence-specifically.

Materials and methods
Production and purification of truncated HepK
Unable to produce catalytically active, intact HepK (GenBank accession no. U68034) by expression in *Escherichia coli*, we produced a truncated, water-soluble version of HepK that lacks two presumptive transmembrane regions, as follows. A 3'-terminal portion of *hepK* encoding residues 267-575 of HepK was amplified by PCR with primers 5'-GGAATTCCATATGCG-GACTAGTCGAGCGATCGCT-3' and 5'-CGGGGATCCTAACTTTGCTC-CTGAAGTG-3' (introduced *Nde*I and *Bam*HI sites are underlined). The PCR product was cloned between the *Nde*I and *Bam*HI sites of plasmid pET-14b (Novagen, Inc., Madison, WI), which provided an N-terminal hexa-histidine tag. The resulting plasmid was de-noted pRL2406, and the tagged, truncated product was denoted H_6-'HepK. DNA sequencing confirmed that the PCR product was error-free. To overproduce H_6-'HepK, *Escherichia coli* strain BL21 (DE3) transformed with pRL2406 was grown in 500 ml LB medium supplemented with 100 µg ml^{-1} ampicillin at 37°C to an OD_{600} of 0.5-0.6, 0.5 mM isopropyl-β-D-thiogalactopyranoside was added, and incubation was continued for 5 h. H_6-'HepK was purified to homogeneity as follows: *E. coli* suspended in 50 mM Na phosphate, 100 mM NaCl, pH 7.0, was broken with a French press (American Instrument Co. Div. Travenol Laboratories, Inc., Silver Spring, MD). The supernatant solution from centrifugation for 20 min at 20,000 x *g* at 4°C was applied to a cobalt-based resin column (Clontech Laboratories, Inc., Palo Alto, CA), which was then washed thrice with 50 mM Na phosphate, 300 mM NaCl, 5 mM imidazole, pH 7.0. H_6-'HepK eluted with 50 mM Na phosphate, 300 mM imidazole, pH 5.5, was loaded onto a Sephadex G-100 gel-filtration column (Amersham Pharmacia Biotech Inc., Piscataway, NJ) from which it was eluted at 4°C with 50 mM Na phosphate, 100 mM NaCl, pH 7.0, at 20 ml h^{-1}. Hexa-histidine-tagged DevR (H_6-DevR) and hexa-histidine-tagged mutant 'HepK (H_6-'HepK-H348A) were prepared as described previously [7].

Gel mobility shift assay *in vitro* for DNA-protein complexes
Mobility shift assays [19] were performed with a "DIG Gel Shift Kit" (Roche Molecular Biochemicals, Indianapolis, IN) according to [20]. The DNA probes used in these experiments are shown in **Figure 1**. The DNA probes were prepared by PCR with the following primers: A1, 5'-GCTCTAGAATTAGGTTTATCC-3' and 5'-GAAATGGATTGAATACAATCCTC-3'; B2, 5'-CCGAATTC-TACAGAGCTTTGTTTCTCAG-3' and 5'-GATATGAATTCGACAAC-CTAATTTTTAAC-3'; X1, 5'-ATCGATTTTTTAACATAAATTGCC-3' and 5'-TACTTGTTTACTTGCACAATAATTTTC-3'; X12, 5'-CTATGACTATT-TAAAGAG-3' and 5'-TACTTGTTTACTTGCACAATAATTTTC-3'; X2, 5'-CAAAGGTTAGGTTAATTGCC-3' and 5'-CGATTTTTAAAATCAG-TATCC-3', and X13, 5'-TCCACAATATTTAAGTATTTTAT-3' and 5'-GGATATAAGACTCTGTAGAAATG-3'. The DNA fragments were 3'-end-labeled by using DIG-11-ddUTP and terminal transferase. Labeled DNA probes (0.4-0.8 ng) were incubated with 0.3 µg of purified H_6-'HepK or H_6-'HepK-H348A (protein) in binding buffer (25 mM HEPES, pH 7.9, 5 mM $MgCl_2$, 25 mM NaCl, 0.5 mM DTT, 5% glycerol, 5 µg BSA) containing 0.5 µg of poly-(dI-dC/dI-dC) in a final volume of 20 µl. For gel shift competition, H_6-'HepK or H_6-'HepK-H348A was bound with at least 75-fold excess (75- to 270-fold molar ratios based on the molecular weight of the competitor DNAs) of unlabeled competitor DNA for 15 min at room temperature before addition of the probe. Incubation with the probe lasted for 20 min. The mixture was then loaded on an 8% polyacrylamide gel (30:1 acrylamide-bisacrylamide) in Tris-glycine buffer (50 mM Tris, 380 mM glycine, 2 mM EDTA, pH 8.5 [19]) that had been pre-run for 1 h at 4°C and 140 V. Electrophoresis was performed at 4°C in a Tris-glycine buffer, for 2 to 2.5 h at a constant voltage of 140 V (ca. 30 mA). The gel was then electroblotted onto a Hybond-N+ Nylon membrane
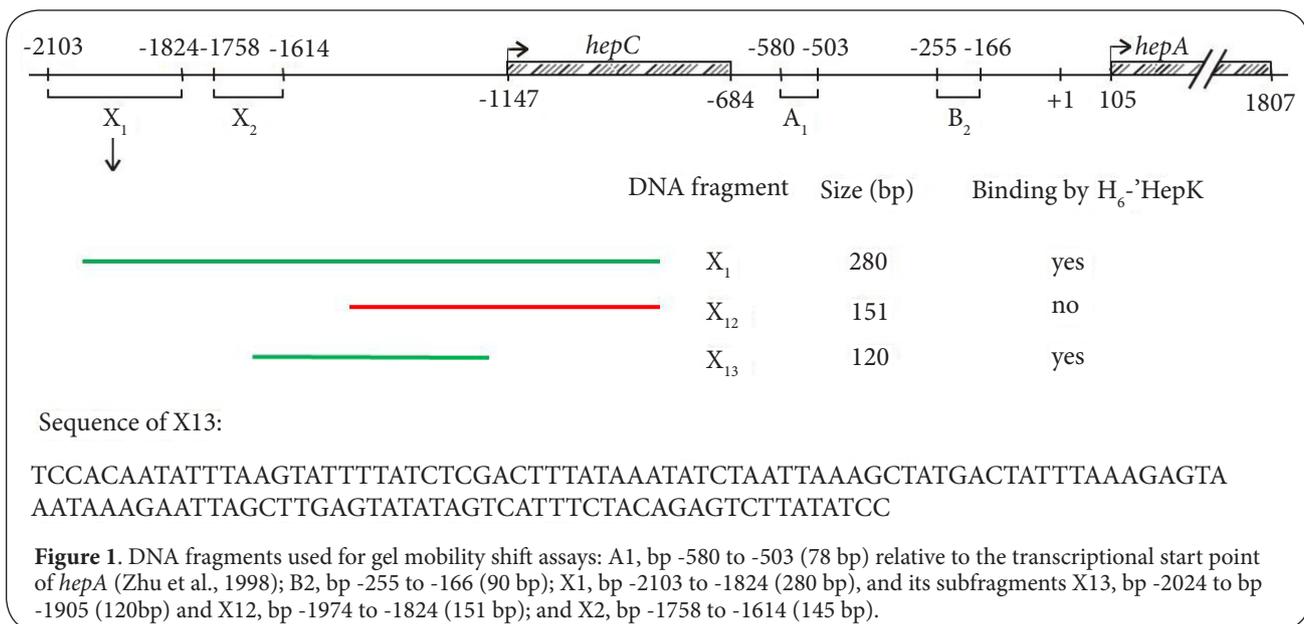
Figure 1. DNA fragments used for gel mobility shift assays: A1, bp -580 to -503 (78 bp) relative to the transcriptional start point of *hepA* (Zhu et al., 1998); B2, bp -255 to -166 (90 bp); X1, bp -2103 to -1824 (280 bp), and its subfragments X13, bp -2024 to bp -1905 (120bp) and X12, bp -1974 to -1824 (151 bp); and X2, bp -1758 to -1614 (145 bp).

(Amersham Pharmacia) at 200 mA in the same Tris-glycine buffer at 4℃ for 1 h, the membrane baked at 80℃ for 2h, and chemiluminescence detected according to the manufacturer's instructions.

Results

Recombinant H_6-'HepK, overexpressed in *E. coli* and purified to homogeneity (**Figure 2A**, lane 3), binds fragment X1 (**Figure 3A**, lane 2), and also X13 (**Figure 3B**, lane 2), a subfragment of X1 (**Figure 1**), but not fragments X2, B2, A1 and X12 (**Figure 3A**, lanes 3-10 and **Figure 1**). Competition experiments with unlabeled A1 showed that a 75-fold excess of A1 (by mass; 270-fold molar excess) does not significantly affect the formation of a complex between X1 and H_6-'HepK (**Figure 3C**, lanes 3 and 5), whereas a 75-fold excess of X1 reduces complex-formation by approximately 90% (**Figure 3C**, lanes 4 and 3). Phosphorylation of H_6-'HepK is labile to acid and stable to alkali, supporting the idea that its site of auto-phosphorylation is a histidine residue [7,22]; the H348A substitution abolishes autophosphorylation [7]; and (unphosphorylated) H_6-'HepK-H348A binds X1specifically (**Figure 4**, lane 2). Competition experiments with unlabeled A1 (**Figure 4**, lane 4) and unlabeled X1 (**Figure 4**, lane 3) showed similar results to that of wild-type H_6-'HepK (**Figure 3C**, lanes 5 and 4). These results provide additional information that the specific binding does not require phosphorylation. The control experiment, the purified H_6-DevR protein (**Figure 2B**, lane 3) produced no mobility shift of X1 (**Figure 3C**, lane 2), showing that the site-specific binding by H_6-'HepK is not due to the hexa-histidine tag.
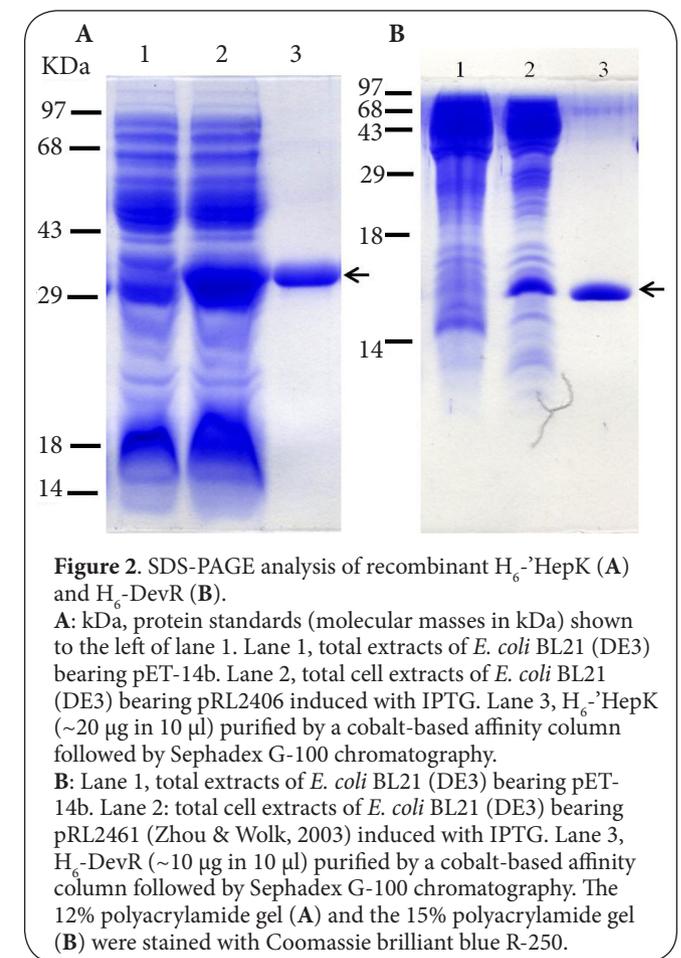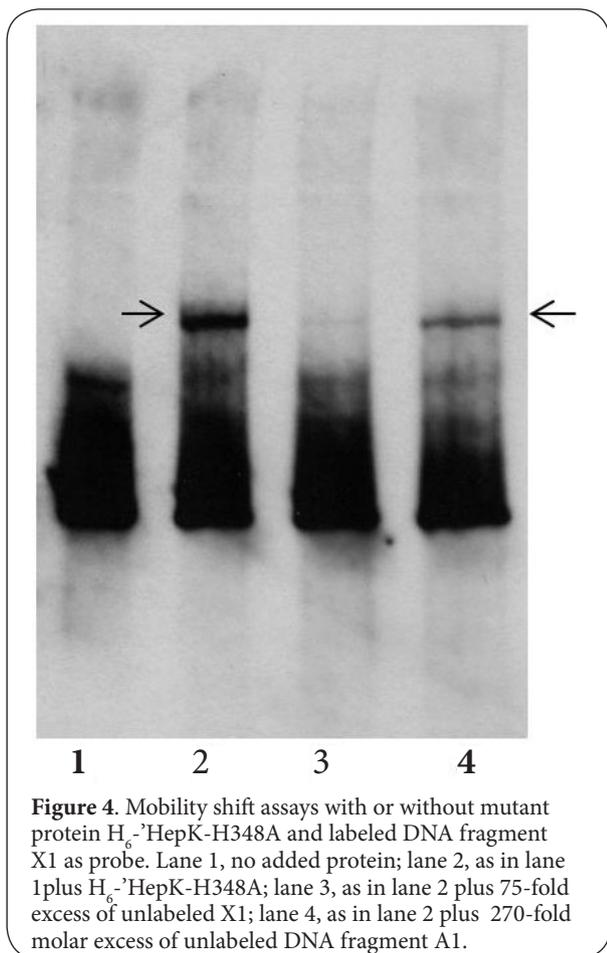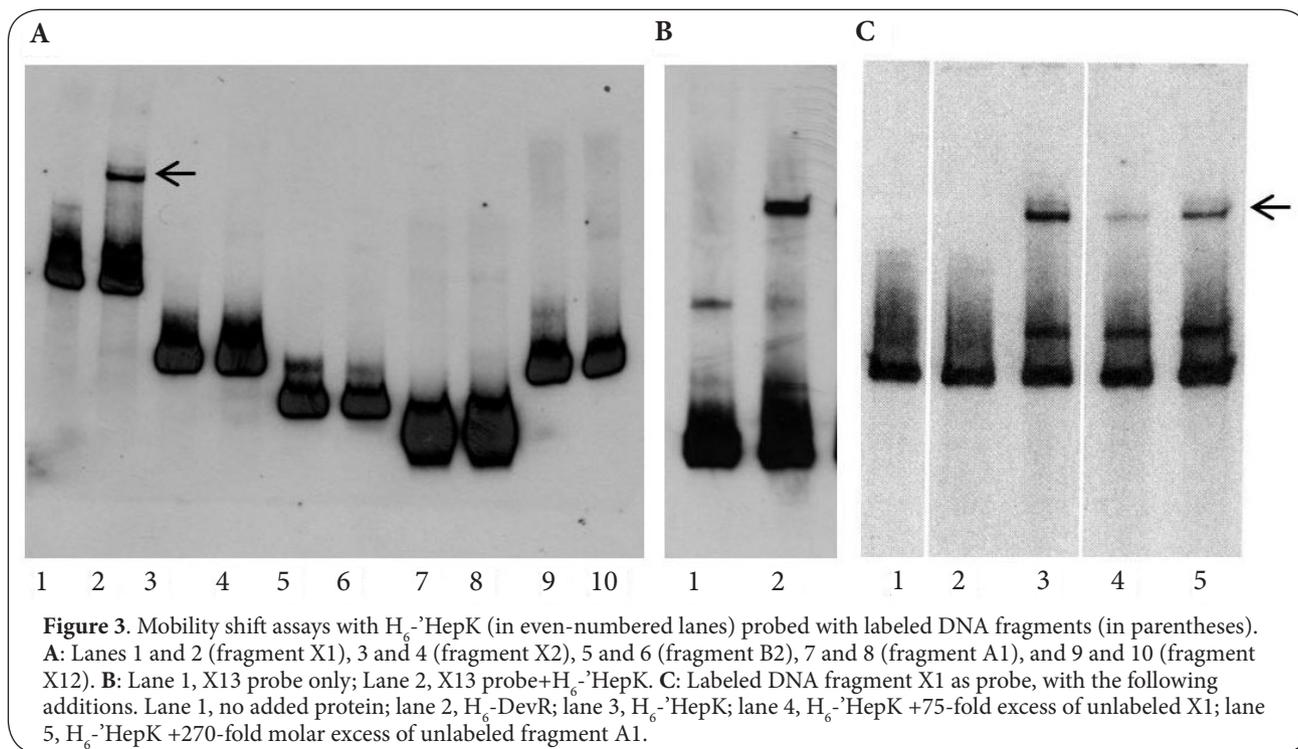

Figure 2. SDS-PAGE analysis of recombinant H_6-'HepK (**A**) and H_6-DevR (**B**).
A: kDa, protein standards (molecular masses in kDa) shown to the left of lane 1. Lane 1, total extracts of *E. coli* BL21 (DE3) bearing pET-14b. Lane 2, total cell extracts of *E. coli* BL21 (DE3) bearing pRL2406 induced with IPTG. Lane 3, H_6-'HepK (~20 μg in 10 μl) purified by a cobalt-based affinity column followed by Sephadex G-100 chromatography.
B: Lane 1, total extracts of *E. coli* BL21 (DE3) bearing pET-14b. Lane 2: total cell extracts of *E. coli* BL21 (DE3) bearing pRL2461 (Zhou & Wolk, 2003) induced with IPTG. Lane 3, H_6-DevR (~10 μg in 10 μl) purified by a cobalt-based affinity column followed by Sephadex G-100 chromatography. The 12% polyacrylamide gel (**A**) and the 15% polyacrylamide gel (**B**) were stained with Coomassie brilliant blue R-250.

Discussion

Several bacterial proteins with high homology to histidine kinases are apparently not kinases [1]. For example, *Azotobacter vinelandii* NifL contains five conserved blocks characteristic

Figure 3. Mobility shift assays with H_6-'HepK (in even-numbered lanes) probed with labeled DNA fragments (in parentheses). **A**: Lanes 1 and 2 (fragment X1), 3 and 4 (fragment X2), 5 and 6 (fragment B2), 7 and 8 (fragment A1), and 9 and 10 (fragment X12). **B**: Lane 1, X13 probe only; Lane 2, X13 probe+H_6-'HepK. **C**: Labeled DNA fragment X1 as probe, with the following additions. Lane 1, no added protein; lane 2, H_6-DevR; lane 3, H_6-'HepK; lane 4, H_6-'HepK +75-fold excess of unlabeled X1; lane 5, H_6-'HepK +270-fold molar excess of unlabeled fragment A1.

Figure 4. Mobility shift assays with or without mutant protein H_6-'HepK-H348A and labeled DNA fragment X1 as probe. Lane 1, no added protein; lane 2, as in lane 1plus H_6-'HepK-H348A; lane 3, as in lane 2 plus 75-fold excess of unlabeled X1; lane 4, as in lane 2 plus 270-fold molar excess of unlabeled DNA fragment A1.

of histidine sensor kinases, including the conserved His residue [23], but purified NifL has not been observed to autophosphorylate [21,24] nor is that residue required for *in vivo* function of NifL [25]. However, HepK evidently is an autokinase: purified H_6-'HepK autophosphorylates highly efficiently *in vitro* [7]. Some transcriptional activators, e.g., GAL4 [26], NtrC [27,28] and Spo0A [29] bind DNA independent of their state of phosphorylation, but activate transcription efficiently only when phosphorylated. Purified, intact HepK, complete with its two presumptive transmembrane domains [10], had no detectable autokinase activity *in vitro* [7]. It is possible that the two presumptive transmembrane domains of intact HepK *in vivo* are responsible for sensing a signal specific to heterocyst development, and subsequently trigger autokinase activity of HepK *in vivo*. Although the soluble, truncated, recombinant form of HepK binds DNA independent of phosphorylation, the biological consequences of that binding may depend on whether HepK is phosphorylated. ToxR is a transmembrane transcriptional activator with a cytoplasmic DNA-binding domain [30]. Because truncated HepK binds site-specifically to DNA but has no known DNA-binding motif, it may be a prototype for a new class of protein-histidine kinase that, like ToxR, serves also as a membrane-tethered transcription factor, which may also require an intramembrane proteolytic activation [31-33] in response to a signal specific to heterocyst development.

Genomic sequence data have identified no presumptive protein histidine kinase genes in animal genomes including human genome [34,35]. Since some two-component phos-

phorelay proteins are essential for the viability, virulence, and drug resistance of microbial pathogens including human fungal and bacterial pathogens [36-39], novel anti-microbial drugs targeted to protein histidine kinase in two-component phosphorelay systems may prove high specificity and minimal toxicity [40,41]. Several series of inhibitors to bacterial histidine kinase have been reported in the literature [42-44], however, most appear to suffer from high hydrophobicity, poor selectivity, and excessive protein binding and/or limited bioavailability [45]. The strong hydrophobicity of these molecules makes formulation and drug delivery impossible. Unlike these compounds, small DNA molecules bound specifically by a protein-histidine kinase HepK are able to bypass the drawbacks of conventional inhibitors. These sequence-specific small DNA molecules have several unique advantages in developing of novel anti-microbial drugs, such as high solubility, high specificity, minimal toxicity, efficient synthesis, easy of formulation and delivery. Therefore, our finding provides an exciting opportunity for developing small DNA molecules as novel anti-microbial drugs.

Conclusion

We have shown that although lacking a known DNA-binding motif, a truncated, soluble version of sensory protein-histidine kinase HepK binds sequence-specifically to a fragment of DNA found upstream from *hepC*, a gene that is required for the synthesis of heterocyst envelope polysaccharide of cyanobacteria. The conserved phosphorylation histidine residue of HepK kinase is not required for this DNA-binding activity. Therefore, regulation of the synthesis of heterocyst envelope polysaccharide by HepK may be, at least in part, independent of two-component phosphorylation. For our best knowledge it is the first example of a protein histidine kinase without a known DNA binding motif that binds DNA sequence-specifically. Our finding may enable development of small DNA molecule as highly specific anti-microbial drugs because protein histidine kinases are broadly conserved in microbial pathogens but absent in humans.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Authors' contributions	RZ	OK
Research concept and design	✓	✓
Collection and/or assembly of data	✓	✓
Data analysis and interpretation	✓	✓
Writing the article	✓	✓
Critical revision of the article	✓	✓
Final approval of article	✓	✓
Statistical analysis	--	--

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