THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 279, No. 51, Issue of December 17, pp. 53078–53086, 2004
Printed in U.S.A.

Five Histidine Kinases Perceive Osmotic Stress and Regulate Distinct Sets of Genes in *Synechocystis**

Received for publication, September 3, 2004, and in revised form, October 7, 2004
Published, JBC Papers in Press, October 7, 2004, DOI 10.1074/jbc.M410162200

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Microorganisms respond to hyperosmotic stress via changes in the levels of expression of large numbers of genes. Such responses are essential for acclimation to a new osmotic environment. To identify factors involved in the perception and transduction of signals caused by hyperosmotic stress, we examined the response of *Synechocystis* sp. PCC 6803, which has proven to be a particularly useful microorganism in similar analyses. We screened knockout libraries of histidine kinases (Hiks) and response regulators (Rres) in *Synechocystis* by DNA microarray and slot-blot hybridization analyses, and we identified several two-component systems, which we designated Hik-Rre systems, namely, Hik33-Rre31, Hik34-Rre1, and Hik10-Rre3, as well as Hik16-Hik41-Rre17, as the transducers of hyperosmotic stress. We also identified Hik2-Rre1 as a putative additional two-component system. Each individual two-component system regulated the transcription of a specific group of genes that were responsive to hyperosmotic stress.

A number of microorganisms respond to environmental stress by exploiting two-component systems (1) that consist of a histidine kinase (Hik)[1] and a response regulator (Rre). Histidine kinases contain a sensing domain and a Hik domain, and, in response to a change in environmental conditions, a conserved histidine residue in the Hik domain is phosphorylated with a phosphate group from ATP (1). The phosphoryl group is then transferred to a conserved aspartate residue in the receiver domain of the response regulator. The resultant modulation of this domain mediates transfer of the signal to the signaling pathway (2).

In *Escherichia coli*, the EnvZ-OmpR two-component system responds to an increase in external osmolarity (3). EnvZ is a histidine kinase that is autophosphorylated with a phosphate group from ATP at a conserved histidine residue. The phosphoryl group is then transferred to a conserved aspartate residue in OmpR, the cognate response regulator (4). However, the genome of *Synechocystis* does not encode any protein that is homologous to EnvZ. A second two-component system involved in the transduction of osmotic signals in *E. coli* is the KdpD-KdpE system (5). KdpD consists of four transmembrane domains, with sequence motifs typical of sensor kinases in the C-terminal region and a transmitter domain in the N-terminal region. KdpE is the cognate response regulator of KdpD, and ~30% of the amino acid sequence of KdpE is identical to that of OmpR. The proteins Hik20 (Sll1590) and Rre19 (Sll1592) of *Synechocystis* are strongly homologous to KdpD and KdpE of *E. coli*, respectively. We postulated that additional two-component systems might exist for the perception of hyperosmotic stress and the transduction of the signal.

Characterization of the entire complement of two-component systems that are involved in the perception and transduction of hyperosmotic signals, requires systematic and genome-wide analysis. *Synechocystis* is particularly suitable for such analysis, since it is easily manipulated; it responds to hyperosmotic stress; and its genome contains 3661 putative genes of which 47 are genes for Hiks and 45 are genes for Rres (6, 7) (see also www.kazusa.or.jp/cyanobase/Synechocystis/index.htm). Such analysis is facilitated by the availability of high quality cDNA microarrays (8).

In our efforts to identify two-component systems that are involved in the perception and transduction of hyperosmotic signals, we screened knockout libraries of Hiks and Rres by RNA slot-blot and genome-wide DNA microarray analyses. We identified four two-component systems and an additional potential two-component system.

EXPERIMENTAL PROCEDURES

Strains and Culture Conditions—A glucose-tolerant (GT) strain of *Synechocystis* sp. PCC 6803 was kindly provided by Dr. J. G. K. Williams (Du Pont de Nemours, Wilmington, DE), and a glucose-sensitive (PCC) strain was obtained from Professor S. Shestakov (Department of Genetics, Moscow State University, Russia). The Hik gene knockout library was produced as described previously (9) (see also www.kazusa.or.jp/cyanobase/Synechocystis/mutants/). The gene knockout library of response regulators was produced on two genetic backgrounds of GT and PCC, as described below (see also www.kazusa.or.jp/cyanobase/Synechocystis/mutants/). GT cells were grown photoautotrophically at 34 °C in BG-11 medium buffered with 20 mM

* This work was supported, in part by Grants-in-aid for Scientific Research no. 14086207 (to N. M.) and no. 16013249 (to I. S.) from the Ministry of Education, Science, Sports, and Culture of Japan; and no. 13854002 (to N. M. and I. S.) from the Japan Society for Promotion of Science) and by Grants no. 04-04-48200 (to V. V. Z.) and no. 03-04-48581 (to D. A. L.) from the Russian Foundation for Basic Research. This work was also supported by the Program for Cooperative Research on Stress Tolerance of Plants of the National Institute for Basic Biology, Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: Hik, histidine kinase; Rre, response regulator; WT, wild type; ORF, open-reading frame.

HEPES-NaOH (pH 7.5) under continuous illumination from incandescent lamps at 70 μmol of photons/m^2/s, with aeration by air that contained 1% CO_2, as described previously (10), and were served as wild-type cells. Mutant cells were grown under the same conditions as wild-type cells, except in the case of precultures, in which BG-11 medium was supplemented with an antibiotic (20 μg/ml spectinomycin or 25 μg/ml kanamycin for cells in which the genome had been mutated by insertion of a spectinomycin-resistance gene cassette or a kanamycin-resistance gene cassette, respectively). For exposure of cells to hyperosmotic stress, cells in a 50-ml culture were grown under standard conditions for 16 h (when the optical density at 730 nm of the culture was 0.3), and then 5.0 M sorbitol was added to the culture to give a final concentration at 0.5 M. The duration of incubation under osmotic stress was 20 min.

Construction of the Gene Knockout Library of Response Regulators—All lines of cells with mutated response regulators were constructed by gene-targeted mutagenesis, which was designed on the basis of the nucleotide sequence of the gene for each response regulator (6, 7). Fragments of DNA, corresponding to the partial or complete sequence of the gene for each response regulator, were amplified by PCR and then cloned into an appropriate vector. The gene for each response regulator was disrupted by insertion of a cassette into the coding region or replacement of part of the coding region with a spectinomycin-resistance or kanamycin-resistance gene cassette. The antibiotic-resistance gene cassette was inserted into the coding region of each respective gene in one of two ways. Either the cassette was inserted at a specific restriction site in the coding region or the cassette was transposed into the coding region by the Tn5 transposition system of an EZ::TN^{TM}<KAN-2> Insertion kit (Epicenter Technologies, Madison, WI), according to the manufacturer's instructions. *Synechocystis* cells were transformed with plasmids as described by Williams (11). Details of the construction of mutants can be found in the Cyanobase (www.kazusa.or.jp/cyanobase/Synechocystis/mutants/).

Southern Blotting Analysis—DNA was extracted from wild-type and ΔRre mutant cells as described by Williams (11). After cells had been treated with a saturated solution of NaI and then with lysozyme, the genomic DNA was extracted with phenol, purified with a mixture of phenol, chloroform, and isoamyl alcohol (25:24:1, v/v) and precipitated with ethanol. Approximately 2 μg of total DNA were digested for 16 h with 10 units of a restriction enzyme, such as BlnI and MunI (TaKaRa Bio Co. Ltd., Ohtsu, Japan), and the resultant fragments were separated on an 0.8% agarose gel. The DNA fragments were transferred to a Hybond-N$^+$ nylon membrane (Amersham Biosciences) by capillary transfer and then allowed to hybridize to specific probes. DNA fragments bounded by PCR primers indicated by the *small black arrowheads* in Fig. 3*A* were amplified by PCR and used as probes. We used primers *rre31*-f (5′-GTTAGAAAGTGGAGTAATGG-3′) and *rre31*-r (5′-CAAAGGTAGACCGTATAAAG-3′) for amplification of the gene for Rre31, primers *rre1*-f (5′-AGGGACGATTTTACCTAAGAATCC-3′) and *rre1*-r (5′-ACCTTGGGAAAAATCAAAAAAGAC-3′) for Rre1, primers *rre17*-f (5′-CCAGGCTAACATCAACAAAA-3′) and *rre17*-r (5′-CTTGGGCGATTACAAACTTA-3′) for Rre17, and primers *rre3*-f (5′-GGAGGCGGCCTTGATTGCA-3′) and *rre3*-r (5′-CATCCTTCAAAGCCTTCTG-3′) for Rre3. Labeling, hybridization, and washing were performed as described in instructions supplied with the AlkPhos Direct Labeling and Detection System with CDP-star (Amersham Biosciences). DNA fragments were conjugated with alkaline phosphatase (Alkphos Direct kit). After hybridization and washing, blots were soaked in CDP-star solution (Amersham Biosciences), and signals from hybridized transcripts were detected with a luminescence image analyzer (LAS-1000; Fuji-Photo Film, Tokyo, Japan).

Isolation of mRNA—After incubation of cultures under designated conditions, 50-ml aliquots were rapidly combined with an equal volume of ice-cold ethanol that contained 5% (w/v) phenol for instantaneous killing of cells to prevent degradation of mRNA. After collection of the killed cells by centrifugation at 1,000 × *g* for 5 min at 4 °C, total RNA was isolated by the hot phenol method as described previously (12). The extracted RNA was treated with DNase I (Nippon Gene, Tokyo, Japan) to remove contaminating DNA and then purified with a mixture of phenol, chloroform, and isoamyl alcohol (25:24:1, v/v) and precipitated in ethanol.

RNA Slot-Blot Hybridization and Northern Blotting—For RNA slot-blot hybridization (13) 10 μg of total RNA were applied to a Hybond-N+ nylon membrane. The RNA on the membrane was allowed to hybridize with a specific probe that had been generated from a hyperosmotic stress-inducible gene, such as *fabG* (*sll0330*), *slr1544*, *hspA* (*sll1514*), *dnaK2* (*sll0170*), *slr0967*, or *htrA* (*slr1204*).

For Northern blotting, 15 μg of total RNA were fractionated by electrophoresis on a 1.2% agarose gel that contained 2.05 M formaldehyde. The RNA was transferred to a Hybond-N$^+$ nylon membrane by capillary transfer and allowed to hybridize with a specific probe. Labeling of the probe, hybridization, and washing were performed as described for Southern blotting analysis. Blots were also probed with the gene for subunit B of RNase P as a control.

DNA Microarray Analysis—*Synechocystis* DNA microarrays (CyanoCHIP) were purchased from TaKaRa Bio Co. Ltd. (Ohtsu, Japan), and DNA microarray analysis was performed as described previously (14, 15). All experiments were performed with CyanoCHIP version 1.6, which included 3074 out of the 3264 genes on the *Synechocystis* chromosome, and results were quantified with the IMAGENE version 5.5 program (BioDiscovery, El Segundo, CA). Changes in the levels of transcripts of individual genes relative to the total level of mRNA were calculated after normalization by reference to the total intensity of signals from all genes with the exception of genes for rRNAs.

RESULTS

Gene Regulation in Response to Hyperosmotic Stress Involves a Family of Hiks That Includes Hik33, Hik34, Hik16, and Hik41—Earlier work from our laboratory demonstrated that exposure of *Synechocystis* to hyperosmotic stress caused by 0.5 M sorbitol, which reduces the growth rate by 50%, enhanced the expression of 113 genes with induction factors higher than 2.0 (15). Perception of hyperosmotic stress involved a histidine kinase, Hik33, and other unknown components (16). We also demonstrated that salt stress caused by 0.5 M NaCl was perceived by Hik33, Hik34, and a combination of Hik16 and Hik41 (17). The set of stress-inducible genes differed between salt stress and osmotic stress (15) but we postulated that both types of stress might share common sensors and response regulators. With this possibility in mind, we examined the effects of inactivation in Hik33, Hik34, Hik16, and Hik41 on hyperosmotic stress-inducible gene expression.

Hyperosmotic stress-inducible genes that had induction factors higher than 4.0 and that were affected by inactivation, separately, of Hik33, Hik34, Hik16, and Hik41 are listed in Table I. The effects of these mutations on gene expression can be divided into four groups, in terms of the genes that are affected. Group 1 contains *fabG* and 10 other genes. Mutation of Hik33 greatly reduced the inducibility by hyperosmotic stress of genes in this group.

Group 2 includes 19 genes, whose induction by hyperosmotic stress was depressed in ΔHik34 cells. These genes include *hspA* for a small heat-shock protein, *clpB1* for ClpB protease, *sodB* for superoxide dismutase, *htpG* for heat-shock protein 90, *dnaK2* for heat-shock protein 70, *dnaJ* for heat-shock protein 40, *groEL2* for chaperonin 60–2, *groEL1* for chaperonin 60–1, *groES* for chaperonin 10, and other genes for proteins of known and unknown function.

Group 3 contains genes whose expression was not affected in ΔHik33 and in ΔHik34 mutant cells but was suppressed almost to background levels in ΔHik16 and in ΔHik41 cells. This group also includes two genes for proteins of unknown function, *sll0939* and *slr0967*.

Group 4 contains genes whose induction of expression was unaffected by inactivation of any of the examined Hiks. This group includes the *rlpA* and *repA* genes for rare lipoprotein A, *htrA* for a serine protease, and 17 other genes.

The DNA microarray studies for which results are summarized in Table I indicated that 32 of 52 hyperosmotic stress-inducible genes, with induction factors higher than 4.0, are under the control of Hik33, Hik34, or Hik16 and Hik41. However, 20 of the hyperosmotic stress-inducible genes were regulated by as yet unknown mechanism(s) that might not involve any Hiks. We also examined the effects of mutation of Hik20 on the induction of gene expression by hyperosmotic stress. We found that Hik20 did not regulate the hyperosmotic stress-inducibility of the expression of any genes.

TABLE I

Hyperosmotic stress-inducible genes in WT cells and effects of the inactivation of Hik33, Hik34, Hik16, Hik41, and Hik10 on their induction under hyperosmotic stress

Cells, grown under control conditions, were incubated with 0.5 M sorbitol for 20 min. Each value indicates the ratio of the level of the transcript in stressed cells to that in control cells. The numbering of ORFs corresponds to that of Kaneko *et al.* (6). Three independent and two independent experiments were performed with wild-type and mutant cells, respectively, and essentially the same results were obtained in the replicates. Table I lists the hyperosmotic stress-inducible genes with induction factors higher than 4.0 in wild-type cells. The total list can be accessed at www.genome.ad.jp/kegg/expression/.

ORF	Gene	Product	Induction by 0.5 M sorbitol					
			WT	ΔHik33	ΔHik34	ΔHik16	ΔHik41	ΔHik10
Group 1: Gene whose induction by hyperosmotic stress was diminished or significantly reduced in ΔHik33 cells								
sll1483		Periplasmic protein	39.1	4.0	26.7	18.7	20.4	42.0
sll0330[a]	*fabG*	3-Ketoacyl-ACP reductase	32.7	2.9	39.8	29.5	27.1	54.1
slr1544		Putative protein	22.3	1.3	20.6	15.1	11.5	23.9
ssl2542	*hliA*	High light-inducible protein	12.4	1.2	9.0	9.8	7.4	8.4
ssr2595	*hliB*	High light-inducible protein	11.4	1.1	10.7	14.2	8.1	13.6
ssr2016		Putative protein	8.1	1.2	5.9	12.5	3.8	12.5
ssl1633	*hliC*	High light-inducible protein	7.4	1.0	8.3	9.5	6.8	11.3
ssl3446		Putative protein	5.4	1.6	3.3	7.0	5.4	9.6
slr0381	*gloA*	Lactoylglutathione lyase	4.6	1.8	5.0	5.7	3.4	6.2
sll2012	*sigD*	RNA polymerase sigma factor	4.3	1.7	5.3	5.1	3.0	4.9
sll1541		Lignostilbene-α,β-dioxygenase	4.3	1.6	3.3	3.3	3.1	4.3
Group 2: Gene whose induction by hyperosmotic stress was diminished or significantly reduced in ΔHik34 cells								
sll1514	*hspA*	Small heat-shock protein	87.7	74.7	16.4	94.6	106.3	82.3
sll0846		Putative protein	25.1	13.6	4.6	19.5	16.2	18.7
slr1963		Water-soluble carotenoid protein	17.3	11.4	4.5	15.5	23.2	14.7
slr1641	*clpB1*	ClpB protein	17.2	12.6	2.9	18.3	13.6	20.5
slr0959		Putative protein	10.1	7.1	3.8	13.3	7.2	12.1
slr1516	*sodB*	Superoxide dismutase	9.3	7.3	3.2	8.3	8.6	9.9
sll0430	*htpG*	Heat-shock protein 90	9.3	6.2	1.9	10.3	7.1	9.0
sll1884		Putative protein	8.9	5.9	3.3	7.2	7.2	8.4
slr1603		Putative protein	8.9	6.5	3.4	8.5	9.0	9.0
slr1915		Putative protein	8.4	5.1	2.8	8.9	6.6	10.5
ssl2971		Putative protein	7.6	4.2	1.9	5.3	5.0	7.0
slr1285	*hik34*	Sensor histidine kinase	7.5	5.2	0.8	8.5	7.9	11.9
slr1413		Putative protein	7.1	3.9	2.5	6.4	5.8	8.1
sll0170	*dnaK2*	Heat-shock protein 70	6.8	5.9	2.5	6.5	5.8	8.3
sll0005	*spkH*	Serine/threonine kinase	6.1	3.2	1.6	4.7	3.6	4.9
slr2076	*groEL1*	60-kDa chaperonin 1	6.0	1.9	1.6	3.7	4.9	3.5
slr0093	*dnaJ*	Heat-shock protein 40	5.2	3.0	1.5	5.7	4.5	4.9
slr2075	*groES*	10-kDa chaperonin	5.2	3.1	1.5	4.6	5.4	4.0
sll0416	*groEL2*	60-kDa chaperonin 2	5.0	4.3	2.0	4.3	4.9	5.5
Group 3: Gene whose induction by hyperosmotic stress was diminished or significantly reduced in ΔHik16 and ΔHik41 cells								
sll0939		Putative protein	9.1	4.3	4.7	2.1	1.1	11.2
slr0967		Putative protein	7.0	3.7	3.4	2.0	1.1	6.1
Group 4: Gene whose induction by hyperosmotic stress was unaffected in ΔHik33, ΔHik34, ΔHik16 or ΔHik41 cells								
sll1863		Putative protein	50.4	32.4	37.7	36.1	53.4	56.6
sll1862		Putative protein	45.6	32.0	38.6	28.3	45.6	38.9
sll0528		Putative protein	40.2	8.5	16.6	31.8	33.9	40.3
slr1204	*htrA*	Serine protease HtrA	16.0	15.2	29.4	21.7	16.3	1.2
slr0423	*rlpA*	Rare lipoprotein A	14.6	8.4	12.9	7.8	13.0	11.6
sll1722		Putative protein	12.6	7.8	8.1	4.3	6.1	5.4
sll0306	*sigB*	RNA polymerase sigma factor	9.7	8.3	6.6	10.1	8.5	11.0
slr0581		Putative protein	6.4	4.7	4.7	3.2	4.0	3.7
ssr1853		Putative protein	6.3	4.7	3.8	2.7	3.5	3.7
slr1119		Putative protein	6.0	3.0	3.2	5.8	5.9	5.4
slr0852		Putative protein	5.0	3.4	2.4	4.8	4.3	6.3
ssr3188		Putative protein	4.6	3.9	2.7	4.0	3.9	4.0
slr0112		Putative protein	4.6	3.8	3.6	2.5	2.6	3.9
sll0294		Putative protein	4.5	2.7	2.8	4.1	4.3	3.8
slr0895		Transcriptional regulator	4.4	6.4	7.5	9.0	8.2	6.3
ssl3177	*repA*	Rare lipoprotein A	4.3	3.6	5.1	4.0	2.8	4.7
sll1085	*glpD*	Glycerol-3-P dehydrogenase	4.3	4.8	4.1	3.8	7.4	5.4
slr1051		Putative protein	4.3	4.3	4.5	4.3	4.9	4.6
sll0293		Putative protein	4.0	4.3	4.7	3.6	4.6	4.2
sll0470		Putative protein	4.0	3.1	3.0	2.3	2.6	3.2

[a] Underlining indicates genes that were used as probes for screening the Rre knockout library by RNA slot-blot hybridization.

Rre31, Rre1, and Rre17 Are the Potential Cognate Response Regulators of Hik33, Hik34, Hik16 and Hik41—In prokaryotic two-component signal transduction systems, Hik is phosphorylated in response to a stimulus and the resultant phosphoryl group is transferred to a cognate response regulator. After phosphorylation, this response regulator transmits the signal to the pathway that ultimately affects gene expression. In order to identify candidates for Rres that are located downstream of Hik33, Hik34, Hik16, and Hik41, we screened a mutant library of Rres by RNA slot-blot hybridization analysis using, as probes, the *fabG*, *hspA*, and *slr0967* genes, whose expression in response to hyperosmotic stress was controlled by Hik33, Hik34, and Hik16 plus Hik41, respectively. Fig. 1 shows representative results. Among the Rre mutants, only ΔRre31 failed in the induction by hyperosmotic stress of the *fabG* gene, as was the case for ΔHik33 cells. In all the other lines of ΔRre mutant cells, the induction by hyperosmotic stress of this gene was equivalent to that in wild-type cells (Fig. 1*A*). These ob-

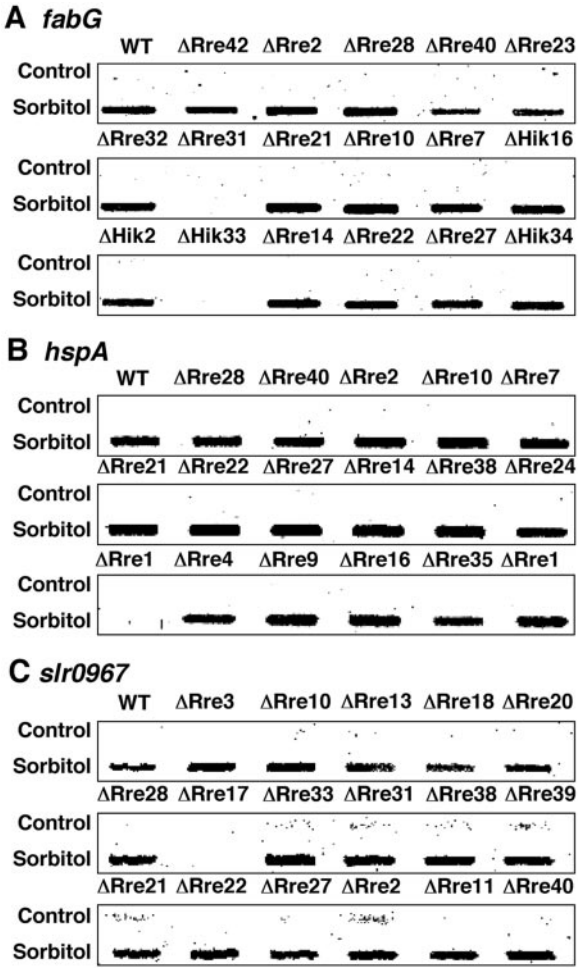

FIG. 1. **RNA slot-blot analysis of the hyperosmotic stress-inducible expression of the *fabG*, *hspA*, and *slr0967* genes in ΔRre mutants.** Total RNA was isolated from cells before (*Control*) and 20 min after the start of treatment with 0.5 M sorbitol (*Sorbitol*). 10-μg aliquots of total RNA were transferred to membranes and allowed to hybridize with DNA probes that corresponded to the *fabG* gene (*A*), the *hspA* gene (*B*) and the *slr0967* gene (*C*).

servations suggest that Rre31 is a candidate for the cognate Rre of Hik33. When the *hspA* gene was used as the probe for the expression of genes in group 2, ΔRre1 was negative for induction by hyperosmotic stress, whereas all the other lines of Rre mutant cells were clearly positive for induction of the *hspA* gene (Fig. 1*B*), suggesting that Rre1 is a candidate for the cognate Rre of Hik34. Similar experiments, performed with the *slr0967* gene as probe, suggested that Rre17 is a candidate for the cognate Rre of Hik16 and Hik41 (Fig. 1*C*).

We examined the involvement of these Rres in signal transduction upon exposure of cells to hyperosmotic stress in further detail by monitoring hyperosmotic stress-inducible gene expression by DNA microarray analysis. Table II shows that the group 1 hyperosmotic stress-inducible genes, whose expression was controlled by Hik33, were also under the control of Rre31. Rre1 regulated the expression of the hyperosmotic stress-inducible genes in group 2, which were also regulated by Hik34. However, we also noted that the expression of the *sigB* gene and of some genes for putative proteins, such as *sll0528*, was diminished in ΔRre1 mutant cells but not in ΔHik34 cells (Table II). These observations suggest that there might be another Hik that perceives hyperosmotic stress and transfers that signal to Rre1. Analysis of this Hik is described below. In the case of the hyperosmotic stress-inducible genes in group 3, which were controlled by Hik16 and Hik41, we found that the induction of gene expression was also controlled by Rre17 (Table II).

Induction by Hyperosmotic Stress of htrA Is Regulated by Rre3—The expression of a number of hyperosmotic stress-inducible genes in group 4 (Table I) was unaffected by mutation of the above-described Rres. Therefore, we rescreened the Rre mutant library by RNA slot-blot hybridization using, as probes, the *htrA, rlpA,* and *sll1862* genes, all of which are in group 4. We also used, as a probe, the *ggpS* (*sll1566*) gene for glucosylglycerol phosphate synthase, which is the key enzyme in the synthesis of a compatible solute glucosylglycerol (18). The *ggpS* gene revealed a hyperosmotic induction factor of 3.4, and belongs to group 4. Our results demonstrated that the expression of *htrA* was abolished in cells with a mutation in Rre3, while expression of the other three genes was unaltered in any of the other lines of Rre mutant cells upon exposure to hyperosmotic stress (data not shown).

The nucleotide sequences of plasmids in *Synechocystis* (7) suggests the presence of three extra two-component systems, with genes for a Hik and a Rre located in close proximity in each case. We examined the genome-wide expression of hyperosmotic stress-inducible genes in cells with mutations in these Hiks using DNA microarrays. Our results indicated that inactivation of none of the three plasmid-encoded Hiks affected the inducibility by hyperosmotic stress of gene expression. Our results suggest that the three two-component systems encoded by the plasmids are not involved in the perception of hyperosmotic stress.

Hik2 and Hik10 Interact with Rre1 and Rre3, respectively, as Potential Cognate Partners in the Hyperosmotic Stress-induced Expression of Genes—In order to identify potential candidates for Hiks that perceive hyperosmotic stress and transfer the signal to Rre1 and Rre3, we exploited the yeast two-hybrid system, using Rre1 and Rre3 as bait. We identified Hik2 and Hik10 as components that interact with Rre1 and Rre3, respectively. DNA microarray analysis revealed that Hik10 regulated the hyperosmotic stress-inducible expression of the *htrA* gene (Table I). This gene was also regulated by Rre3 (Table II), suggesting that Hik10 plus Rre3 constitute a two-component system that regulates the expression of the *htrA* gene.

The complete replacement of the *hik2* gene by the mutated *hik2* gene was not achieved in ΔHik2 cells; only 30% of the native copies of the *hik2* were replaced by the mutated gene (see www.kazusa.or.jp/cyanobase/Synechocystis/mutants/). The incomplete replacement of wild-type genes by mutant genes might explain why the phenotype of ΔHik2 cells, with respect to the hyperosmotic stress-inducible expression of *sigB* and four other genes, was apparently unchanged from the wild type. However, it is very likely that the interaction between Hik2 and Rre1 is relatively strong because Hik2 and Rre1 in bait clones were "captured" by Rre1 and Hik2 encoded by prey vectors, respectively. Therefore, it seems plausible that Hik2 is a factor that is located upstream of Rre1 in the signal transduction pathway.

The Hik33-Rre31, Hik34-Rre1, Hik16-Hik41-Rre17, and Hik10-Rre3 Systems Regulated the Expression of Different Sets of Genes under Hyperosmotic Stress—DNA microarray analysis indicated that the hyperosmotic stress-inducible expression of genes in group 1, such as *fabG* and *slr1544*, was regulated by the Hik33-Rre31 two-component system. We confirmed the contribution of Hik33 and Rre31 to the response of these genes by Northern blotting. Fig. 2*A* shows that the capacity for induction by hyperosmotic stress was abolished in both ΔHik33 and ΔRre31 mutant cells but not in ΔHik34 and ΔRre1 mutant cells. From among the hyperosmotic stress-inducible genes in

TABLE II

Hyperosmotic stress-inducible genes in WT cells and effects of the inactivation of Rre31, Rre1, Rre17, and Rre3 on their induction by hyperosmotic stress

Cells, grown under control conditions, were incubated with 0.5 M sorbitol for 20 min. Each value indicates the ratio of the level of the transcript in stressed cells to that in control cells. The numbering of ORFs corresponds to that of Kaneko *et al*. (6). Three independent and two independent experiments were performed with wild-type and mutant cells, respectively, and essentially the same results were obtained in the replicates. The total list can be accessed at www.genome.ad.jp/kegg/expression/.

ORF	Gene	Product	Induction by 0.5 M sorbitol					Induction affected by:				
			WT	ΔRre31	ΔRre1	ΔRre17	ΔRre3	ΔHik33	ΔHik34	ΔHik16	ΔHik41	ΔHik10
Gene whose induction by hyperosmotic stress was diminished or significantly reduced in DRre31 cells												
sll1483		Periplasmic protein	39.1	10.9	23.6	21.5	71.9	X[a]	-[b]	-	-	-
sll0330	*fabG*	3-Ketoacyl-ACP reductase	32.7	4.2	18.2	15.3	62.1	X	-	-	-	-
slr1544		Putative protein	22.3	1.3	8.0	21.5	18.5	X	-	-	-	-
ssl2542	*hliA*	High light-inducible protein	12.4	1.9	4.3	11.9	8.1	X	-	-	-	-
ssr2595	*hliB*	High light-inducible protein	11.4	1.8	6.5	14.4	14.0	X	-	-	-	-
ssr2016		Putative protein	8.1	1.7	4.3	9.1	11.6	X	-	-	-	-
ssl1633	*hliC*	High light-inducible protein	7.4	3.1	5.4	7.8	7.4	X	-	-	-	-
ssl3446		Putative protein	5.4	1.7	2.1	8.0	7.1	X	-	-	-	-
slr0381	*gloA*	Lactoylglutathione lyase	4.6	1.9	5.6	4.9	9.3	X	-	-	-	-
sll2012	*sigD*	RNA polymerase sigma factor	4.3	1.9	3.3	6.8	5.8	X	-	-	-	-
sll1541		Lignostilbene-α,β-dioxygenase	4.3	1.7	2.9	3.9	3.3	X	-	-	-	-
Gene whose induction by hyperosmotic stress was diminished or significantly reduced in ΔRre1 cells												
sll1514	*hspA*	Small heat-shock protein	87.7	84.6	2.6	65.4	63.2	-	X	-	-	-
sll0528[c]		Putative protein	40.2	8.9	2.3	43.0	57.7	-	-	-	-	-
sll0846		Putative protein	25.1	11.7	1.1	13.9	14.4	-	X	-	-	-
slr1963		Water-soluble carotenoid protein	17.3	9.6	3.7	18.3	16.8	-	X	-	-	-
slr1641	*clpB1*	ClpB protein	17.2	10.4	0.9	23.4	15.5	-	X	-	-	-
slr0959		Putative protein	10.1	7.4	1.1	9.0	6.8	-	X	-	-	-
sll0306	*sigB*	RNA polymerase sigma factor	9.7	8.6	3.3	12.0	14.4	-	-	-	-	-
slr1516	*sodB*	Superoxide dismutase	9.3	9.1	1.3	9.2	8.3	-	X	-	-	-
sll0430	*htpG*	HtpG, heat-shock protein 90	9.3	6.7	1.5	10.6	7.5	-	X	-	-	-
sll1884		Putative protein	8.9	4.7	1.5	9.8	7.4	-	X	-	-	-
slr1603		Putative protein	8.9	5.0	1.3	10.7	9.8	-	X	-	-	-
slr1915		Putative protein	8.4	4.9	1.1	9.2	7.6	-	X	-	-	-
ssl2971		Putative protein	7.6	3.0	1.1	6.7	4.3	-	X	-	-	-
slr1285	*hik34*	Sensor histidine kinase	7.5	4.6	1.2	7.6	9.4	-	X	-	-	-
slr1413		Putative protein	7.1	3.5	1.2	6.7	4.6	-	X	-	-	-
sll0170	*dnaK2*	Heat-shock protein 70	6.8	4.6	0.9	8.7	9.4	-	X	-	-	-
sll0005	*spkH*	Serine/threonine kinase	6.1	3.2	0.9	3.6	2.8	-	X	-	-	-
slr2076	*groEL1*	60-kDa chaperonin 1,	6.0	3.2	1.1	4.1	2.2	-	X	-	-	-
slr1119		Putative protein	6.0	3.5	2.1	4.1	4.7	-	-	-	-	-
slr0093	*dnaJ*	Heat-shock protein 40	5.2	2.9	0.8	4.6	3.4	-	X	-	-	-
slr2075	*groES*	10-kDa Chaperonin	5.2	2.9	1.1	4.1	3.4	-	X	-	-	-
slr0852		Putative protein	5.0	2.4	1.3	5.0	4.1	-	-	-	-	-
sll0416	*groEL2*	60-kDa chaperonin 2	5.0	4.1	1.6	5.6	6.7	-	X	-	-	-
ssr3188		Putative protein	4.6	3.0	1.4	5.4	3.7	-	-	-	-	-
Gene whose induction by hyperosmotic stress was diminished or significantly reduced in ΔRre17 cells												
sll0939		Putative protein	9.1	4.0	4.0	1.1	7.5	-	-	X	X	-
slr0967		Putative protein	7.0	3.1	2.7	1.2	4.4	-	-	X	X	-
Gene whose induction by hyperosmotic stress was diminished in ΔRre3 cells												
slr1204	*htrA*	Serine protease HtrA	16.0	24.8	9.2	9.8	1.1	-	-	-	-	X
Gene whose induction by hyperosmotic stress was unaffected in ΔRre31, ΔRre1, ΔRre17 or ΔRre3 cells												
sll1863		Putative protein	50.4	17.7	38.9	42.7	49.5	-	-	-	-	-
sll1862		Putative protein	45.6	10.2	21.7	45.2	30.0	-	-	-	-	-
slr0423	*rlpA*	Rare lipoprotein A	14.6	4.7	12.4	8.5	11.5	-	-	-	-	-
sll1722		Putative protein	12.6	3.3	3.8	3.9	3.2	-	-	-	-	-
slr0581		Putative protein	6.4	3.2	3.0	3.5	2.9	-	-	-	-	-
ssr1853		Putative protein	6.3	2.9	3.5	2.6	2.1	-	-	-	-	-
slr0112		Putative protein	4.6	2.4	3.6	3.5	3.0	-	-	-	-	-
sll0294		Putative protein	4.5	2.6	2.5	2.1	2.2	-	-	-	-	-
slr0895		Transcriptional regulator	4.4	5.2	4.1	5.9	4.1	-	-	-	-	-
ssl3177	*repA*	Rare lipoprotein A	4.3	2.0	4.2	5.2	4.0	-	-	-	-	-
sll1085	*glpD*	Glycerol-3-phosphate dehydrogenase	4.3	4.4	4.8	3.7	5.5	-	-	-	-	-
slr1501		Putative protein	4.3	3.3	3.7	3.3	3.0	-	-	-	-	-
sll0293		Putative protein	4.0	2.8	3.8	3.5	3.2	-	-	-	-	-
sll0470		Putative protein	4.0	2.1	3.1	2.6	2.4	-	-	-	-	-

[a] X indicates genes whose expression was induced by treatment with 0.5 M sorbitol for 20 min (see Table I) but whose expression under these conditions was inhibited by mutation of the indicated Hik.

[b] - indicates genes whose expression was induced by treatment with 0.5 M sorbitol for 20 min (see Table I) and whose expression under these conditions was not affected by mutation of the indicated Hik.

[c] Underlining indicates genes whose induction by hyperosmotic stress was controlled by Rre1 but not by Hik34.

group 2 that are controlled by Hik34 and Rre1, we selected the *hspA* and *dnaK2* genes for confirmation of the contributions of Hik34 and Rre1. Northern blotting (Fig. 2*A*) showed that the extent of induction of the expression of these genes was significantly reduced in ΔHik34 and in ΔRre1 mutant cells, indicating that the Hik34-Rre1 two-component system regulated the hyperosmotic stress-inducible expression of the *hspA* and *dnaK2* genes.

We also examined the induction by hyperosmotic stress of the expression of the *slr0967* gene in ΔHik16, ΔHik41, and ΔRre17 mutant cells by Northern blotting. Our results showed clearly that induction of the expression of the *slr0967* gene was

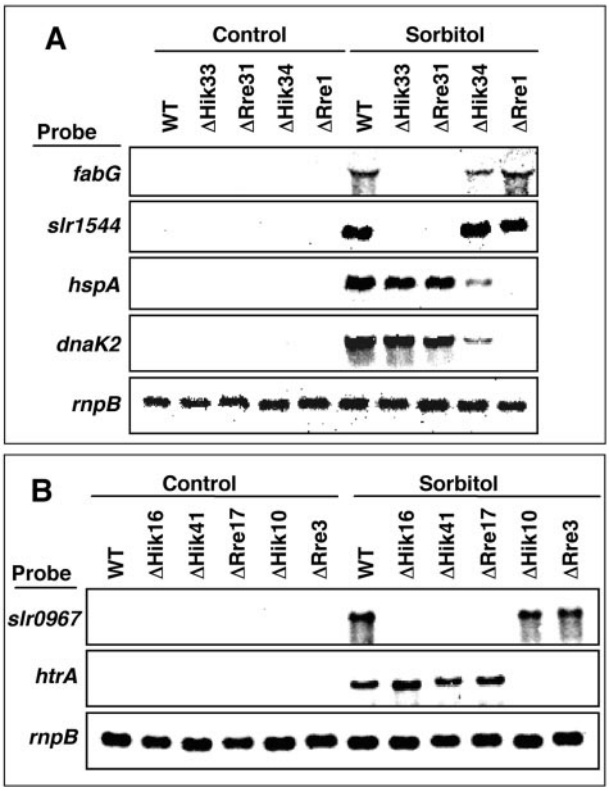

FIG. 2. **Northern blotting analysis of the hyperosmotic stress-inducible expression of genes.** *A*, the expression of *fabG*, *slr1544*, *hspA* and *dnaK2* genes in wild-type (*WT*), ΔHik33, ΔRre31, ΔHik34, and ΔRre1 mutant cells. *B*, the expression of *slr0967* and *htrA* genes in WT, ΔHik16, ΔHik41, ΔRre17, ΔHik10, and ΔRre3 mutant cells. Total RNA was isolated from cells before (*Control*) and 20 min after the start of treatment with 0.5 M sorbitol (*Sorbitol*). Aliquots of 15 μg of extracted RNA were fractionated on a 1.2% agarose gel that contained 2.05 M formaldehyde and bands of RNA were allowed to hybridize with labeled probes, as indicated.

abolished by mutation of Hik16, of Hik41 or of Rre17 (Fig. 2*B*), suggesting that the Hik16-Hik41-Rre17 system regulated the hyperosmotic stress-inducible expression of the *slr0967* gene.

Fig. 2*B* also shows that the induction by hyperosmotic stress of the expression of the *htrA* gene was completely abolished both in ΔHik10 and in ΔRre3 mutant cells. These results suggest that the induction by hyperosmotic stress of expression of the *htrA* gene was controlled by the Hik10-Rre3 two-component system.

Characterization of Rre Mutants by Southern Blotting Analysis—The extent of replacement of each wild-type *hik* gene by the mutated gene has been described elsewhere (9, 17) (see also www.kazusa.or.jp/cyanobase/Synechocystis/mutants/). Here, we evaluated the extent of replacement of *rre* genes by their mutated alleles.

Fig. 3*A* shows the structure of the *Synechocystis* genome in the vicinity of the genes for Rre31, Rre1, Rre17, and Rre3 and the sites at which an antibiotic resistance gene cartridge was inserted in the various mutants. It is clear that the genes for cognate Hiks and Rres are not located in close proximity to each other, with the exception of genes for Hik16 and Rre17. In the ΔRre1, ΔRre17, and ΔRre3 mutants, the respective genes were inactivated by insertion of the kanamycin-resistance gene cassette, whereas in ΔRre31 the gene for Rre31 was inactivated by replacement of part of the gene by an omega spectinomycin-resistance gene cassette.

Fig. 3*B* shows the results of Southern blotting with the target genes as probes, which was performed to examine the extent of replacement of the native copies of each gene by mutated copies of the same gene. Chromosomal DNA from

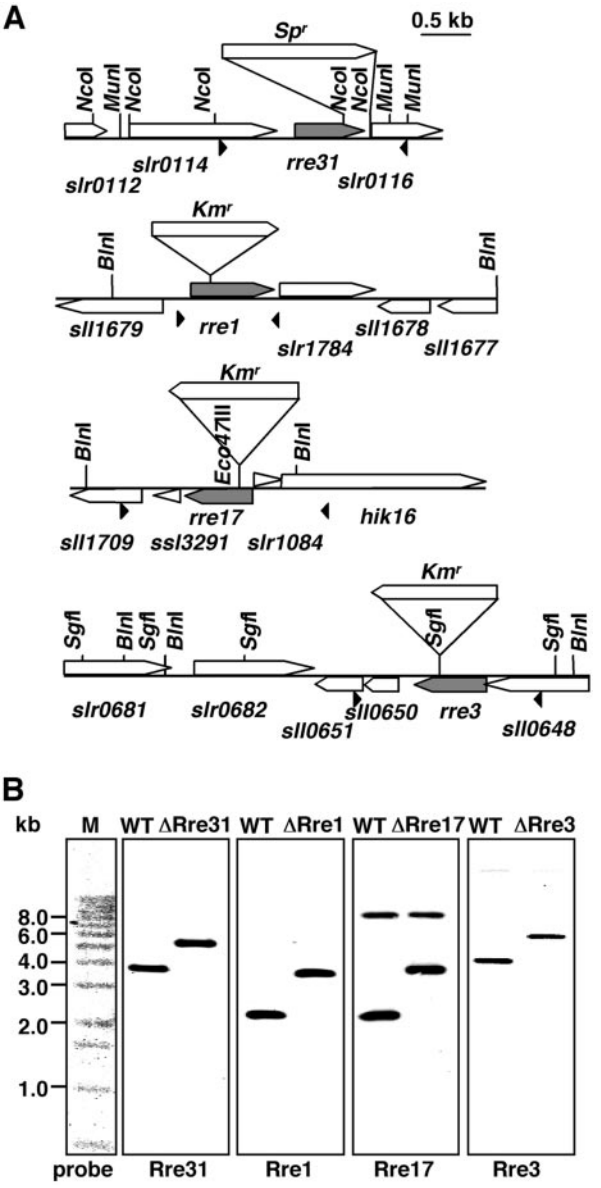

FIG. 3. **Analyses by Southern blotting of the extent of replacement of wild-type copies of the genes for Rres in ΔRre31, ΔRre1, ΔRre17, and ΔRre3 Cells.** *A*, schematic representation of the genotypes of ΔRre31, ΔRre1, ΔRre17, and ΔRre3 cells. Sites and directions of insertions of spectinomycin-resistance and kanamycin-resistance gene cassettes are shown, together with the restriction sites that were used for the insertion of cassettes and Southern blotting analysis. *B*, results of Southern blotting analysis of chromosomal DNA from wild-type (*WT*) and ΔRre mutant cells that had been digested with various restriction enzymes. DNA from ΔRre31 cells was digested with MunI, and DNA from ΔRre1, ΔRre17, and ΔRre3 cells was digested with BlnI. DNA probes that corresponded to the coding sequences of the respective genes for Rres were produced by PCR with forward and reverse primers indicated by *small black arrowheads* in *A*. *Lane M*, size markers.

wild-type and ΔRre31 cells was digested with MunI. In the case of wild-type cells, the probe for *rre31* hybridized with a band of DNA of 3.7 kb, whereas in the case of ΔRre31 it hybridized with a 5.0-kb fragment. Thus, the lengths of hybridizable fragments of DNA from mutant cells had been changed by replacement of part of the gene of interest by the spectinomycin-resistance gene cassette. For analysis of mutations in genes for Rre1, Rre3, and Rre17, we digested chromosomal DNA from wild-type, ΔRre1, ΔRre3, and ΔRre17 cells with BlnI, which cleaves at sites outside the respective coding sequences. In the case of wild-type cells, the probes for *rre1* and *rre3* hybridized with

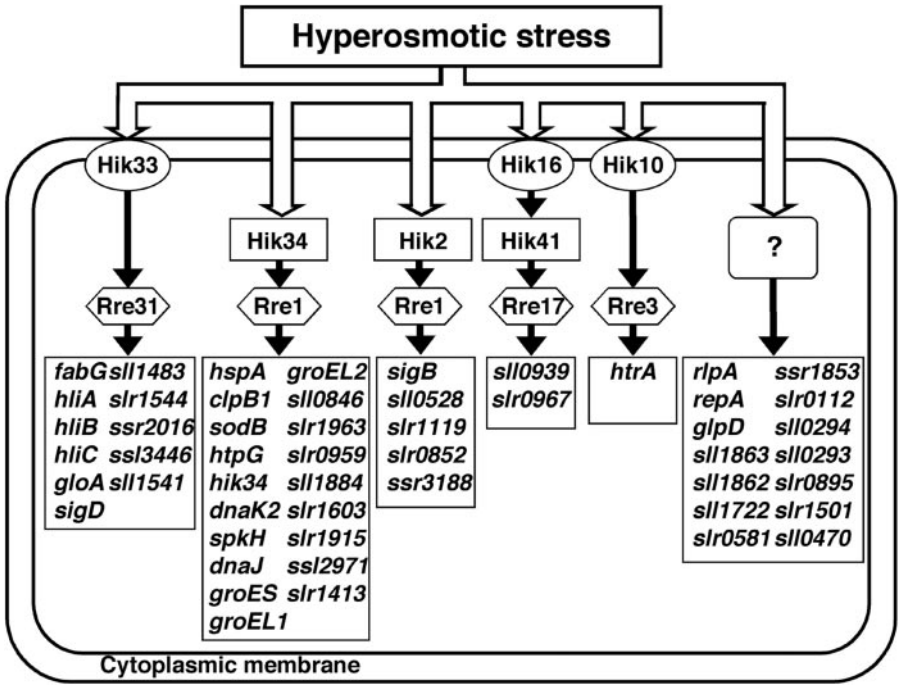

FIG. 4. **A hypothetical scheme for the two-component signal transduction pathways that are activated in response to hyperosmotic stress and the genes whose expression is regulated in the respective pathways.** Because the *hik2* mutation could not be fully segregated, the evidence that supports the placement of Hik2 in this scheme is based on results of the yeast two-hybrid system. Therefore, it differs from the evidence that supports the placement of the other Hik proteins.

2.1-kb and 4.2-kb fragments, respectively, whereas in the case of ΔRre1 and ΔRre3 cells, the DNA fragments were 3.3- and 5.5-kb long, respectively. These results indicated that the lengths of hybridizable fragments had been increased by 1.3 kb and suggested that the kanamycin-resistance gene cassette had been inserted into the *rre1* gene and the *rre3* gene, respectively (Fig. 3*B*). With the probe for the gene for Rre17, we expected to detect two fragments of DNA from wild-type and ΔRre17 cells. A 8.4-kb fragment was detected in the analysis of chromosomal DNA from both wild-type and ΔRre17 cells, whereas a 2.2-kb fragment and a 3.5-kb fragment, which included a 1.3-kb kanamycin-resistance gene cassette, were detected in the analysis of chromosomal DNA from wild-type and ΔRre17 cells, respectively. Fig. 3*B* shows the observed fragments, which were of exactly the expected sizes. These results suggested that the genes for Rre31, Rre1, Rre17, and Rre3 had been mutated in all copies of the chromosome in ΔRre31, ΔRre1, ΔRre17, and ΔRre3 mutant cells.

DISCUSSION

Hyperosmotic Stress: Perception, Transduction of the Signal, and the Genes Regulated in Synechocystis—Systematic mutagenesis of almost all the *hik* and *rre* genes in *Synechocystis*, in combination the genome-wide analysis of gene expression at the transcriptional level, allowed us to identify four Hik-Rre systems and another potential two-component system for the perception of hyperosmotic stress and transduction of the signal. The expression of most of the hyperosmotic stress-inducible genes was controlled by these Hik-Rre systems. Fig. 4 shows the signal transduction pathways that operate when cells are exposed to hyperosmotic stress and the hyperosmotic stress-inducible genes whose expression is controlled by the individual Hik-Rre systems.

The Hik33-Rre31, Hik10-Rre3, and Hik34-Rre1 Two-Component Systems—The Hik33-Rre31 two-component system regulates the inducible expression of eleven genes (Table II). Inactivation of either Hik33 or Rre31 resulted in elimination of or a marked reduction in the hyperosmotic stress-inducible expression of each of these genes. These findings indicate that Hik33 and Rre31 are tightly coupled in the signal transduction pathway. The Hik10-Rre3 two-component system regulates the hyperosmotic stress-inducible expression of only one gene, the *htrA* gene, which encodes a serine protease (Table II). The Hik34-Rre1 two-component system regulates nineteen hyperosmotic stress-inducible genes (Table II). The inducible expression was abolished or significantly reduced by mutation of either Hik34 or Rre1, suggesting the tight coupling of these two components.

The Hik16-Hik41-Rre17 System—The Hik16-Hik41-Rre17 system regulates the hyperosmotic stress-inducible expression of two genes, namely, *sll0939* and *slr0967* (Table II). Inactivation of Hik16, of Hik41 or of Rre17 eliminated the expression of both these genes (Table II), suggesting that Hik16, Hik41, and Rre17 are all essential for the perception of hyperosmotic stress and transduction of the signal that controls the expression of these genes. It seems likely that Hik41 acts downstream of Hik16 since Hik41 is a hybrid-type histidine kinase, which contained both signal receiver domain and histidine kinase domain, whereas Hik16 is a typical Hik with a histidine kinase domain and potential sensory domain that spans the membrane seven times. It is also possible that Hik16 and Hik41 might perceive hyperosmotic stress as a complex. Further studies are necessary to clarify the involvement of these three factors in the sensing of osmotic stress and subsequent signaling.

A Putative Hik2-Rre1 Two-Component System—The expression of the *sigB* gene and four other hyperosmotic stress-inducible genes was controlled by Rre1 but not by Hik34 (Table II). Screening of the Hik mutant library by RNA slot-blot hybridization with a probe derived from the *sigB* gene did not allow us to identify a Hik that might act upstream of Rre1 in the signal transduction pathway that regulates the expression of this gene. Our results suggest that such a Hik might be encoded by a wild-type gene that is incompletely replaced by the corresponding mutated gene in the line of mutant cells. We found evidence for incomplete replacement of the gene for Hik in ΔHik2, ΔHik11, and ΔHik26 cells. The experiments using a yeast two-hybrid system revealed that Hik2 could associate very tightly with Rre1. Therefore, it seems most likely that Hik2 and Rre1 might constitute a two-component system that regulates the expression of *sigB* and four other genes in response to hyperosmotic stress (Table II).

Uncharacterized Pathways of Signals of Hyperosmotic Stress—Our studies with DNA microarrays revealed that expression of fourteen of the fifty-two hyperosmotic stress-inducible genes was not controlled by any of the five Hiks and four Rres discussed above. Screening of the Rre knockout library by slot-blot hybridization with probes derived from genes in this group of fourteen genes, such as *rlpA* and *sll1862*, did not allow us to identify candidates for Rres that might act as signal transducers. However, our Rre knockout library included three mutant lines, ΔRre23, ΔRre25, and ΔRre26, in which the complete replacement of the *rre* gene was not achieved (www.kazusa.or.jp/cyanobase/Synechocystis/mutants/). Thus, possible candidates include Rre23, Rre25 and Rre26. It is more likely that the signals due to hyperosmotic stress that induce the expression of these genes are perceived by unknown mechanisms that are separate from Hik-Rre two-component systems. Such signals might act directly to regulate transcription or the stability of the transcripts of these inducible genes.

Is Rre1 Activated by Two Hiks?—When *Synechocystis* was exposed to hyperosmotic stress, the response regulator Rre1 received signals from two Hiks, namely, Hik34 and another Hik, possibly Hik2, with subsequent regulation of the expression of different sets of genes. The set of genes regulated by Rre1 depended on the Hik that transduced the signal to Rre1. These observations cannot be explained by a two-component system, in which a response regulator regulates the expression of a gene or genes after it receives a signal from the cognate histidine kinase, which is not, itself, directly involved in the control of gene expression. However, the present study suggests that Rre1 might receive a signal from Hik34 and from another Hik, perhaps Hik2. It seems plausible that Hik34 and Hik2 might each associate with Rre1 to form a complex, namely Hik34-Rre1 and Hik2-Rre1, and then these two complexes might act specifically to regulate the expression of different sets of genes.

A similar complex system is found in *Bacillus subtilis*, in which five Hiks, namely, KinA, KinB, KinC, KinD, and KinE, transfer signals (*i.e.* a phosphate residue) to a Rre, Spo0F. However, the five Hik-Rre systems all activate a transcription factor, Spo0A, resulting in the transcriptional repression of the *abrB* gene (19). By contrast, the regulation of gene expression by Rre1 of *Synechocystis* differs from that of *B. subtilis*, because the set of genes under the Hik34-Rre1 system is completely distinct from that putatively under the Hik2-Rre1 system.

Homologs of Hik33, Hik10, Hik16, Hik41, Hik34, and Hik2 in Other Microorganisms—The characteristics of Hik33 have been described previously (9, 16). Histidine kinases that are strongly homologous to Hik33 have been found in all cyanobacteria whose genomes have been sequenced, for example, the sensor kinase NblS in *Synechococcus elongatus* PCC 7942 (58% identity; Ref. 20), the two-component sensor histidine kinase Hik04 (PMM1341) in *Prochlorococcus marinus* MED4 (50% identity; Ref. 21); and signal transduction histidine kinase in *Crocosphaera watsonii* WH 8501 (71% identity; JGI data base at genome.ornl.gov/cgi-bin/JGI_microbial/gene_viewer.cgi?org=cwat&chr=10feb04&contig=Contig197&gene=946). Moreover, Hik33 is also homologous to a putative sensor-like histidine kinase, Ycf26, encoded by the plastid genomes of certain eukaryotic algae, such as *Porphyra purpurea* and *Cyanidium caldarium* (51 and 41% identity, respectively; Ref. 22). In addition, Hik33 has been identified as a sensor of a variety of environmental stresses (14, 16, 17). These findings indicate that Hik33 plays important roles in signaling in cyanobacteria.

The results of an analysis of the amino acid sequence of Hik16 using the TMpred program (www.ch.embnet.org/software/TMPRED_form.html) and a Pfam search (www.sanger.ac.uk/Software/Pfam/search.shtml) suggest that Hik16 contains seven putative transmembrane domains, a GAF domain, a histidine kinase A domain (HisKA) and a histidine kinase-like ATPase (HATPase_C) domain. This sensor of hyperosmotic stress is not strongly homologous to any histidine kinases identified in other organisms with the exception of the osmosensitive K^+ channel histidine kinase in *C. watsonii* WH8501 (44% identity; JGI data base at genome.ornl.gov/cgi-bin/JGI_microbial/gene_viewer.cgi?org=cwat&chr=10feb04&contig=Contig340&gene=3562).

The results of an analysis of the amino acid sequence of Hik10 using the TMpred program suggest that Hik10 contains two putative transmembrane domains. The results of a Pfam search suggest that Hik10 also contains a HAMP domain on the C-terminal side of the second transmembrane domain, as well as HisKA and HATPase_C domains at the C terminus. Hik10 is homologous to histidine kinases in other cyanobacteria, such as the signal-transduction kinase in *C. watsonii* WH8501 (63% identity; JGI data base at genome.ornl.gov/cgi-bin/JGI_microbial/gene_viewer.cgi?org=cwat&chr=10feb04&contig=Contig306&gene=2640), the two-component sensory histidine kinase All4726 in *Anabaena* sp. PCC 7120 (59% identity; CyanoBase data base at www.kazusa.or.jp/cyanobase/Anabaena/cgi-bin/orfinfo.cgi?title=Chr&name=all4726&iden=1) and the signal-transduction histidine kinase in *Nostoc punctiforme* (59% identity; JGI data base at maple.lsd.ornl.gov/cgi-bin/JGI_microbial/gene_viewer.cgi?org=npun&chr=31may01&contig=Contig462&gene=37). Moreover, the domain structure of Hik10 is also homologous to that of proteins in other bacteria, such as the sensor protein TTC1361 in *Thermus thermophilus* HB27 (39% identity) and the sensory histidine kinase BaeS, which forms a two-component regulatory system with BaeR that controls the expression of the *spy* gene for spheroplast protein Y in *E. coli* (36% identity; Ref. 23). However, even though BaeS and Hik10 resemble one another to some extent, they have different functions.

The results of a Pfam search suggest that Hik34 is a unique histidine kinase, with a HisKA domain but no HATPase_C domain of the type that is found in most Hik sensors. This soluble histidine kinase has been shown to act as a sensor of high concentration of NaCl (17). The present study showed that Hik34 is also involved in the sensing of hyperosmotic stress. Proteins homologous to Hik34 have been found in all other cyanobacteria examined, such as *C. watsonii* WH 8501, *Anabaena* sp. PCC 7120, *N. punctiforme*, *Trichodesmium erythraeum* IMS101, and *Thermosynechococcus elongatus* BP-1, an observation that suggests that this histidine kinase may play an important role in signal transduction in all cyanobacteria.

Hik41 is a soluble hybrid-type histidine kinase. It contains a signal receiver domain that is located just upstream of the HisKA domain and a HATPase_C domain (as predicted by a Pfam search). The presence of a signal receiver domain suggests that Hik41 accepts a phosphate group from another Hik. A similar organization of domains is found in other histidine kinases, such as Hik38, Hik40, and Hik42, in *Synechocystis.* However, the Hiks that resemble Hik41 in *Synechocystis* do not appear to be involved in hyperosmotic signal transduction. Hik41-like kinases have been found in other cyanobacteria. For example, *Anabaena* sp. PCC 7120 contains thirteen of this type of histidine kinase and *Gloeobacter violaceus* PCC 7421 contains one Hik41-like histidine kinase, Gll3122. However, homologous Hiks are not encoded in the genomes of *T. elongatus* BP-1 and several marine cyanobacteria. Thus, the functions of the Hiks that resemble Hik41 may have diverged.

In Hik2, another soluble protein, the GAF domain is located just upstream of the HisKA and HATPase_C domains (as pre-

dicted by Pfam). Genes for homologs of Hik2 have been found in all cyanobacteria genomes examined, such as those of *C. watsonii* WH8501, *N. punctiforme*, *Anabaena* sp. PCC 7120, *A. variabilis* ATCC 29413 and *T. elongatus* BP-1. The functions of the Hik2-like proteins in these cyanobacteria have not been reported, but it is possible that such proteins are important for survival.

The various sensors of hyperosmotic stress contain a variety of signal-input domains and they may be located at a variety of sites in the cell; they may perceive hyperosmotic stress in different ways and may transduce the signal to divergent pathways to regulate different sets of genes.

How Do Individual Hiks Perceive Hyperosmotic Stress?—In a previous study we demonstrated that the inducibility by low temperature of cold-inducible genes in *Synechocystis* was enhanced by the genetically engineered rigidification of membrane lipids (24), and the importance of membrane rigidification in the perception of hyperosmotic signals has been postulated by us and by others (25, 26). However, the relationship has not been fully explained, in particular, in terms of the functioning of sensor molecules.

An analysis of predicted amino acid sequence suggests that Hik33 and Hik10 are membrane-bound proteins, each with two transmembrane domains, while Hik16 has seven transmembrane domains. It is possible that some portion of the transmembrane domains of these membrane-bound Hiks perceives the rigidification of membrane lipids that occurs as a result of the shrinkage of *Synechocystis* cells, when these cells are exposed to hyperosmotic stress.

Hyperosmotic stress probably induces several phenomena in addition to the rigidification of membrane lipids (26), for example, a decrease in turgor; increases in intracellular concentrations of ions due to a decrease in cytoplasmic volume; and changes in the hydration states of intracellular proteins. It seems likely that Hik34, a putative Hik (possibly Hik2) and some unknown factors might perceive such signals, with transduction of the signals to pathways that control the expression of a number of genes. Further studies are necessary to clarify these issues.

The Perception of Hyperosmotic Stress and Salt Stress by Individual Hiks—In a previous study (17), we demonstrated that Hik33, Hik34, Hik16, and Hik41 are involved in the perception of salt stress due to 0.5 M NaCl, through regulation of the expression of a small fraction of the total number of salt-inducible genes. In the present study, we demonstrated that Hik33, Hik34, Hik16, and Hik41 are also involved in the perception of hyperosmotic stress, and a major fraction of the hyperosmotic stress-inducible genes is controlled by these Hiks. We demonstrated also that there are genes whose expression was specifically induced by hyperosmotic stress or salt stress (15). For example, the expression of *fabG*, *rlpA*, and *repA* genes was induced by hyperosmotic stress but not by salt stress, whereas the expression of the *pbp* gene for penicillin-binding protein and the *ctpA* gene for C-terminal processing protease was induced by salt stress but not by hyperosmotic stress. Thus, it appear that, even though a similar set of Hiks is involved in the perception of both hyperosmotic stress and salt stress, the genes that are under their control differ under conditions of hyperosmotic stress and salt stress.

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