The EMBO Journal Vol.19 No.6 pp.1327–1334, 2000

The pathway for perception and transduction of low-temperature signals in *Synechocystis*

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Low temperature is an important environmental factor that has effects on all living organisms. Various low-temperature-inducible genes encode products that are essential for acclimation to low temperature, but low-temperature sensors and signal transducers have not been identified. However, systematic disruption of putative genes for histidine kinases and random mutagenesis of almost all the genes in the genome of the cyanobacterium *Synechocystis* sp. PCC 6803 have allowed us to identify two histidine kinases and a response regulator as components of the pathway for perception and transduction of low-temperature signals. Inactivation, by targeted mutagenesis, of the gene for each of the two histidine kinases and inactivation of the gene for the response regulator depressed the transcription of several low-temperature-inducible genes.

Keywords: histidine kinase/low-temperature-inducible gene/response regulator/signal perception and transduction/*Synechocystis*

Introduction

Poikilothermic organisms, such as prokaryotes, plants and fish, sense changes in ambient temperature and acclimate to such changes with greater or lesser efficiency (Kaye and Guy, 1995; Thieringer *et al*., 1998). Numerous low-temperature-inducible genes have been found in a wide range of organisms, e.g. *csp* genes for cold-shock proteins in *Escherichia coli* (Jones *et al*., 1987) and *Bacillus subtilis* (Willimsky *et al*., 1992), *cor* and *cas* genes in plants (Gilmour *et al*., 1992; Wolfraim *et al*., 1993), and genes for fatty acid desaturases in cyanobacteria (Wada *et al*., 1990; Murata and Wada, 1995; Murata and Los, 1997), plants (Gibson *et al*., 1994) and fish (Tiku *et al*., 1996). These low-temperature-inducible genes are thought to play an important role in acclimation to low temperature. To date, however, mechanisms for the perception and transduction of low-temperature signals remain to be characterized.

In a previous study (Vigh *et al*., 1993), we demonstrated that decreases in the degree of unsaturation of fatty acids in the plasma membrane of the cyanobacterium *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*) by catalytic hydrogenation *in vivo* enhanced the expression of the *desA* gene for the Δ12 acyl-lipid desaturase, which is otherwise induced primarily by low temperature. Thus, a change in membrane fluidity appears to be important for the perception of temperature that results in induction of the synthesis of the desaturases (Los *et al*., 1997). However, it remains unclear how a change in membrane fluidity is perceived and how the signal is transduced from the membrane to chromosomes to induce the expression of low-temperature-inducible genes, such as genes for desaturases.

The physical state of the membrane also affects the high-temperature-induced expression of heat-shock genes (Vigh *et al*., 1998). Modification, by genetic manipulation, of the ratio of unsaturated to saturated fatty acids in *Saccharomyces cerevisiae* has a significant effect on the expression of the heat-shock genes *hsp70* and *hsp82* (Carratu *et al*., 1996). These findings support the hypothesis that changes in the state of the membrane might also be important in the regulation of the expression of heat-shock genes.

Physical and chemical stimuli that are generated extra- and intracellularly are perceived by a group of proteins that includes histidine kinases. These proteins are localized on the plasma membrane or in the cytosol in various prokaryotes (Appleby *et al*., 1996), yeast (Maeda *et al*., 1994) and plants (Chang *et al*., 1993; Kakimoto, 1996). It seems likely, therefore, that temperature-induced changes in membrane fluidity might be mediated by a membrane-bound histidine kinase. Kaneko *et al*. (1995, 1996) determined the sequence of the genome of *Synechocystis* and identified 43 putative genes for histidine kinases (Mizuno *et al*., 1996).

In this study we attempted to identify components of the pathway for perception and transduction of low-temperature signals. Among the various low-temperature-inducible genes that had been identified previously, we focused on the *desB* gene for ω3 fatty acid desaturase in *Synechocystis* because the low-temperature-induced expression of this gene has been characterized in detail (Los *et al*., 1997; Los and Murata, 1998). We monitored the promoter response of the *desB* gene to low temperature using a gene for bacterial luciferase as the reporter. Systematic mutagenesis of genes for histidine kinases and random mutagenesis of almost all the genes in the genome allowed us to identify genes for two histidine kinases and a response regulator as components of the pathway for perception and transduction of low-temperature signals.

Results

Systematic mutation of putative genes for histidine kinases in pdesB::lux cells

In order to monitor the inducibility by low temperature of the *desB* gene, we generated a strain of *Synechocystis*,

designated *pdesB::lux*, in which the promoter region of the *desB* gene was ligated to the coding region of the *luxAB* gene for a bacterial luciferase (Los *et al*., 1997). Thus, luciferase activity, monitored in terms of luminescence, could be used as an indicator of low-temperature-inducible changes in the activity of the *desB* promoter.

The products of some of the 43 putative genes for histidine kinases in the genome of *Synechocystis* (Kaneko *et al*., 1995, 1996; Mizuno *et al*., 1996) might plausibly be expected to function as sensors or transducers of environmental or intracellular stimuli (Appleby *et al*., 1996; Mizuno *et al*., 1996). We designated the histidine kinases Hik1–Hik43 and their genes *hik1–hik43*. To investigate the contributions of the various histidine kinases to the induction by low temperature of transcription of the *desB* gene, we attempted to inactivate each of the genes for histidine kinase in *pdesB::lux* cells by inserting a spectinomycin-resistance gene (Sp^r) cassette (Prentki *et al*., 1991) into the coding region or by replacing part of the coding region with the cassette, creating a gene-knockout library.

Each cyanobacterial cell contains >10 copies of the chromosome (Mann and Carr, 1974). Therefore, replacement of the wild-type chromosomes by mutated chromosomes required a lengthy period of time under selective pressure due to spectinomycin in the medium. The 43 lines of transformed cells were cultured for 6 months on agar-solidified BG-11 medium supplemented with 20 μg/ml spectinomycin. If the gene that was the target of the mutation were not essential, we would expect all the copies of the wild-type chromosome eventually to disappear. If the gene were essential, some copies of the wild-type chromosome would be expected to remain. Therefore, using the polymerase chain reaction (PCR), we monitored the extent to which wild-type chromosomes had been replaced by mutated chromosomes (data not shown). The wild-type gene was completely absent from all copies of the chromosome in 33 of our mutant strains. It appeared, therefore, that these 33 *hik* genes were not essential for survival under our growth conditions. Eight *hik* genes were not completely removed. However, the number of copies of wild-type chromosomes relative to the total number of copies of the chromosome was quite small in each case (data not shown). It seemed likely that these eight *hik* genes were important for the growth and survival of *Synechocystis* cells under our culture conditions. We failed to obtain any spectinomycin-resistant cells in our attempts to mutate the *hik13* (sll1003; this nomenclature refers to designations of open reading frames by Kaneko *et al*., 1995, 1996) and *hik15* (sll1353) genes. These two *hik* genes might have been essential for growth under our conditions. Detailed information about these mutants can be found on the web page on which mutants of *Synechocystis* are listed: CyanoMutant, at http://www.kazusa.or.jp/cyano/mutants/.

Inactivation of the hik33 and hik19 genes prevents the induction by low temperature of luciferase activity in pdesB::lux cells

We examined luciferase activity after a decrease in growth temperature in *pdesB::lux* cells and in each line of cells with a mutation in a gene for histidine kinase. Figure 1A shows that the shift in growth temperature

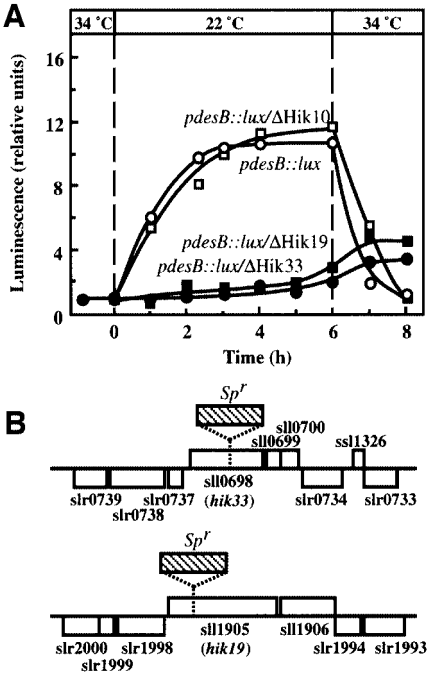

Fig. 1. Temperature-dependent changes in the activity of the *desB* promoter in *pdesB::lux*, *pdesB::lux*/ΔHik10, *pdesB::lux*/ΔHik33 and *pdesB::lux*/ΔHik19 cells, and sites of insertion of the Sp^r cassette in *pdesB::lux*/ΔHik33 and *pdesB::lux*/ΔHik19 cells. (**A**) Cells were grown on agar-solidified medium at 34°C and then transferred to 22°C. Luciferase activity was measured in terms of the intensity of luminescence, as described in Materials and methods. ○, *pdesB::lux*; □, *pdesB::lux*/ΔHik10; ●, *pdesB::lux*/ΔHik33; ■, *pdesB::lux*/ΔHik19. The results are the averages of results of three independent experiments. (**B**) The *Nco*I sites in the *hik33* (sll0698) and *hik19* (sll1905) genes at which the Sp^r cassette (hatched rectangle) was inserted are indicated by dashed vertical lines. Open rectangles indicate open reading frames.

from 34 to 22°C increased the luciferase activity ~10-fold in *pdesB::lux* cells. By contrast, in the two mutants *pdesB::lux*/ΔHik33 and *pdesB::lux*/ΔHik19, in which, respectively, the *hik33* gene and the *hik19* gene had been inactivated by the insertion of the Sp^r cassette, no increase in luciferase activity was observed upon incubation of cells at 22°C (Figure 1A). This result indicated that mutations in the *hik33* and *hik19* genes eliminated the inducibility by low temperature of the *desB* promoter. By contrast, the luciferase activity in other lines with mutant genes for histidine kinase resembled that in *pdesB::lux*; for example, the response of luciferase activity to the shift in temperature in *pdesB::lux*/ΔHik10 cells, in which the *hik10* gene (slr0533) had been inactivated, was the same as that in *pdesB::lux* cells (Figure 1A). The sites of insertion of the Sp^r cassette in *hik33* and *hik19* genes are shown in Figure 1B. Analysis by PCR revealed, however, that the native genes had not been completely eliminated in these lines, suggesting that the genes were essential under our growth conditions.

Expression of low-temperature-inducible genes in wild-type, ΔHik33 and ΔHik19 cells

We next inactivated, separately, the *hik33* and *hik19* genes in wild-type cells to examine the effects of these genes on the expression of low-temperature-inducible

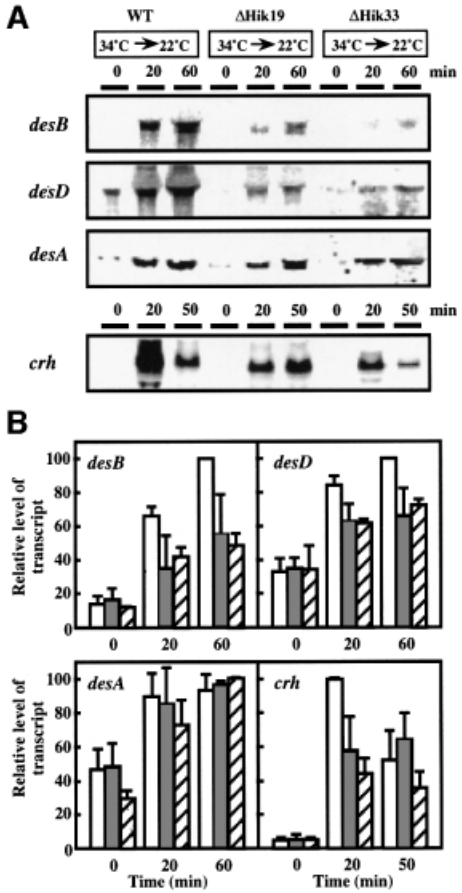

Fig. 2. Induction of *desB*, *desD*, *desA* and *crh* genes in wild-type, ΔHik33 and ΔHik19 cells after a downward shift in temperature. Cells that had been grown at 34°C for 16 h were transferred to 22°C and incubated for the periods of time indicated. Northern blotting analysis of the expression of the *desB*, *desD*, *desA* and *crh* genes was performed as described by Los *et al*. (1997). Total RNA (30 μg for analysis of *des* mRNAs and 5 μg for analysis of *crh* mRNA) was loaded in each lane. (**A**) Northern blots. (**B**) Quantification of transcripts. Open bars, wild-type cells; filled bars, ΔHik19 cells; hatched bars, ΔHik33 cells. The results are the averages from three independent experiments with experimental deviations.

genes. There are four genes for fatty acid desaturases in *Synechocystis*. The *desC* gene is expressed constitutively, while the *desA*, *desB* and *desD* genes are induced after a downward shift in temperature (Los *et al*., 1997). We performed Northern blotting analysis to examine the expression of the *desB*, *desD* and *desA* genes in wild-type, ΔHik19 and ΔHik33 cells before and after a shift in temperature from 34 to 22°C (Figure 2A). As observed previously in wild-type cells (Los *et al*., 1997), the increase in the level of the *desB* transcript was the most conspicuous. There was also a distinct increase in the level of the *desD* transcript. The level of the *desA* transcript increased least of all among the transcripts of the three genes for desaturases. Prior to exposure of cells to 22°C, the levels of the transcripts of the *desB*, *desD* and *desA* genes in the mutant cells were as low as those in wild-type cells (Figure 2A). However, the extent of induction at 22°C of the *desB* and *desD* genes, but not that of the *desA* gene, appeared to be reduced in both ΔHik19 and ΔHik33 mutant cells.

Figure 2B shows the quantitative changes in the levels of the transcripts. The level of the *desB* transcript in wild-type cells was 8-fold higher after incubation for 60 min at 22°C than before the incubation. However, inactivation of the *hik33* gene significantly depressed the low-temperature-induced increase in the level of the *desB* transcript. The low-temperature-induced increase in the level of the *desD* transcript in ΔHik33 cells was also reduced to two-thirds of that in wild-type cells after incubation for 60 min at 22°C. In addition, inactivation of the *hik19* gene depressed the low-temperature-induced enhancement of expression of the *desB* and *desD* genes (Figure 2B). However, the low-temperature-induced enhancement of the expression of the *desA* gene was unaffected by the inactivation of *hik33* and *hik19* (Figure 2B). These results indicated that inactivation of *hik33* and *hik19* suppressed the low-temperature-induced expression of the *desB* and *desD* genes, but not of the *desA* gene.

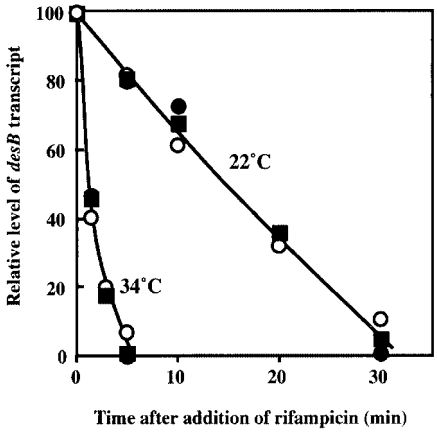

Fig. 3. Dependence on temperature of the stability of *desB* mRNA in wild-type, ΔHik33 and ΔHik19 cells. Cells were grown at 34°C for 16 h and then incubated for 2 h either at 22 or 34°C. An inhibitor of transcription, rifampicin, was added to the cultures at 50 μg/ml at time zero. Aliquots of cultures were withdrawn at the times indicated and levels of *desB* mRNA were determined by Northern blotting analysis. ○, wild-type cells; ●, ΔHik33 cells; ■, ΔHik19 cells. The results are averages from three independent experiments.

The *crh* gene for a homolog of RNA helicase is also a gene that is induced at low temperatures (Chamot *et al*., 1999). Figure 2 shows that the level of *crh* mRNA increased within 20 min after a shift in temperature from 34 to 22°C and then decreased during further incubation at 22°C. The pattern of expression of *crh* mRNA in wild-type cells differed from that of the mRNAs for desaturases. Nonetheless, the increases in the level of *crh* mRNA in 20 min in ΔHik33 and ΔHik19 cells were smaller than those in wild-type cells.

Degradation of desB mRNA in wild-type, ΔHik33 and ΔHik19 cells

In general, the level of an mRNA is regulated by the rate of transcription of the corresponding gene and the stability of the mRNA itself. These factors also control the accumulation of *desB* mRNA when *Synechocystis* cells are exposed to a low temperature (Los *et al*., 1997). Therefore, we compared the stability of *desB* mRNA at 34 and 22°C in wild-type, ΔHik33 and ΔHik19 cells in the presence of an inhibitor of transcription, rifampicin. Figure 3 shows that the rate of degradation of *desB* mRNA was the same in wild-type and mutant cells at both high and low

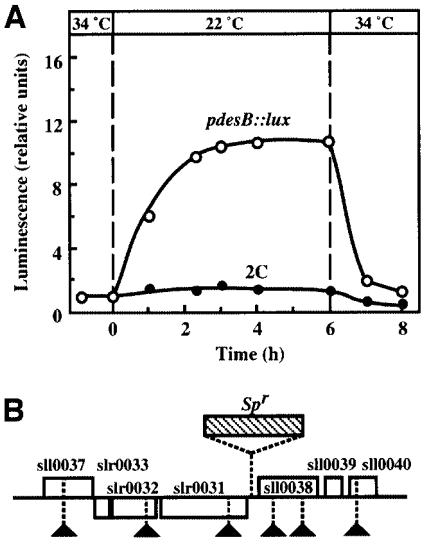

Fig. 4. Changes in the activity of luciferase upon a change in temperature in *pdesB::lux* and 2C cells. (**A**) *pdesB::lux* and 2C cells were grown on agar-solidified medium at 34°C and then transferred to 22°C. Luciferase activity was measured in terms of the intensity of luminescence, as described in Materials and methods. ○, *pdesB::lux*; ●, mutant 2C. The results are the averages of three independent experiments. (**B**) The site of insertion of the Sp^r cassette in the chromosome of mutant 2C cells (hatched rectangle) and the sites of insertion of the Sp^r cassette in wild-type cells (triangles) are indicated. The open reading frame sll0038 corresponds to the response regulator Rer1.

temperatures. The half-life of the *desB* transcript was 15 min at 22°C and 1 min at 34°C. These results indicated that the reduction in the low-temperature-induced accumulation of the *desB* transcript in ΔHik33 and ΔHik19 cells was due to a decrease in the rate of transcription and not to a decrease in the stability of the mRNA, and they suggested, moreover, that both Hik33 and Hik19 might be involved in the low-temperature-induced regulation of transcription of the *desB* gene.

Random mutagenesis and screening of mutants with altered expression of the desB gene

In order to find other components in the pathway for low-temperature signaling, we used random mutagenesis. We introduced the Sp^r cassette randomly into the chromosome of *pdesB::lux* cells by cassette mutagenesis (Hagemann *et al*., 1996). From among ~20 000 spectinomycin-resistant mutants, we isolated 18 mutants in which the response of luciferase activity to a downward shift in temperature was different from that in parental *pdesB::lux* cells (Figure 4). We postulated that in cells of each of these mutant lines a component of the low-temperature signal-transduction pathway might have been mutated. We determined sequences on both sides of the sites of insertion of the cassette in these mutants. We found that in two of the 18 mutants the *hik19* gene for Hik19 had been inactivated by insertion of the Sp^r cassette (data not shown).

In another of these mutant lines, designated 2C, in which the *desB* promoter was not activated by low temperature (Figure 4A), the Sp^r cassette had been inserted in the upstream region of two operons, as shown in Figure 4B. The results suggested that one of the genes in the vicinity of the site of insertion encoded a component of the low-temperature signal-transduction pathway. In order to identify the gene responsible for the elimination of the transcriptional activity, we inactivated five putative genes separately (sll0037, slr0032, slr0031, sll0038, sll0040) in *pdesB::lux* by inserting an Sp^r cassette, as indicated by triangles in Figure 4B. Inactivation of gene sll0038, but not of any of the other genes, depressed the low-temperature-induced increase in luciferase activity in the same way as observed in 2C cells (data not shown). The amino acid sequence deduced from the nucleotide sequence of this gene indicated that its product was one of the 38 response regulators identified in *Synechocystis* (Mizuno *et al*., 1996). We designated the gene *rer1* and its product Rer1.

Inactivation of the rer1 gene in wild-type cells inhibited the induction of the desB gene by low temperature

To examine the role of Rer1 in the regulation of expression of low-temperature-inducible genes, we inactivated the *rer1* gene in wild-type cells by inserting the Sp^r cassette at the *Msc*I site. The extent of the low-temperature-dependent induction of the *desB* transcript was reduced to half that in wild-type cells (Figure 5). By contrast, the inducibility by low temperature of the *desD*, *desA* and *crh* genes was unaffected by the mutation (Figure 5). These results indicated that Rer1 might specifically regulate the expression of the *desB* gene, but not that of the other genes examined.

Discussion

Advantages of using Synechocystis for systematic inactivation of histidine kinases

There are 43 putative genes for histidine kinases in the chromosome of *Synechocystis* (Kaneko *et al*., 1995, 1996; Mizuno *et al*., 1996). Since histidine kinases have been shown, in many cases, to be sensors or components of signal-transducing systems (Appleby *et al*., 1996; Mizuno *et al*., 1996), some of the histidine kinases in *Synechocystis* might also be expected to have similar functions. However, it is impossible to predict the function of each individual histidine kinase on the basis of its primary structure. The functions of ~30 histidine kinases have been identified in *E.coli*, but these enzymes have no obvious counterparts in *Synechocystis* (Mizuno *et al*., 1996).

Using *Synechocystis*, we were able to identify genes for histidine kinases that appear to be involved in the perception and transduction of low-temperature signals even though these genes are essential for survival and cannot be completely eliminated. Each cell of *Synechocystis* contains ~10 identical copies of the chromosome and, thus, essential genes can be inactivated to some extent but not totally with detectable changes in phenotype. The *hik33* and *hik19* genes were not completely eliminated even under selective pressure due to spectinomycin and, therefore, they can be regarded as essential genes. It is impossible to identify genes that might correspond to *hik33* and *hik19* in experiments with *E.coli* or *B.subtilis* because each cell of these bacteria contains only a single copy of the chromosome.

Characteristics of Hik33

The amino acid sequence deduced from the *hik33* gene (sll0698) indicates that Hik33 contains 663 amino acid

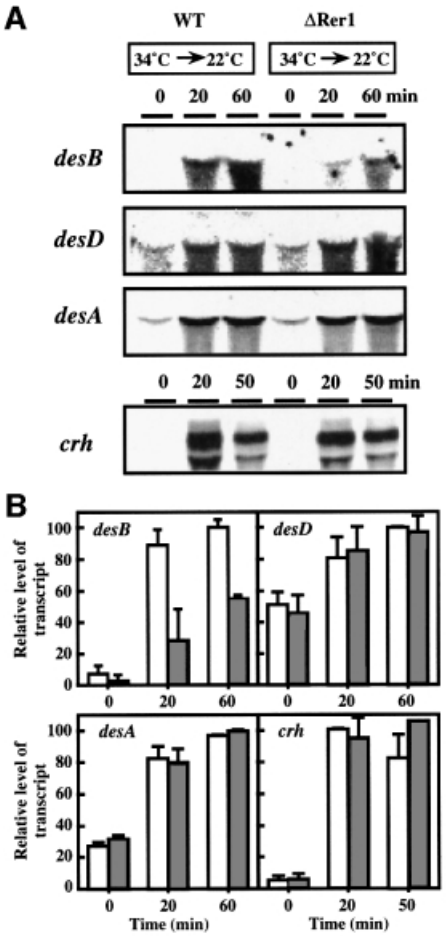

Fig. 5. Induction of *desB*, *desD*, *desA* and *crh* genes in wild-type and ΔRer1 cells after a downward shift in temperature. Cells that had been grown at 34°C for 16 h were transferred to 22°C and incubated for the periods of time indicated. Northern blotting analysis of the expression of *desB*, *desD* and *desA* genes was performed as described by Los *et al*. (1997). Total RNA (30 μg for analysis of *des* mRNAs and 5 μg for analysis of *crh* mRNA) was loaded in each lane. (**A**) Northern blots. (**B**) Quantification of transcripts. Open bars, wild-type cells; filled bars, ΔRer1 cells. The results are the averages of results from three independent experiments with experimental deviations.

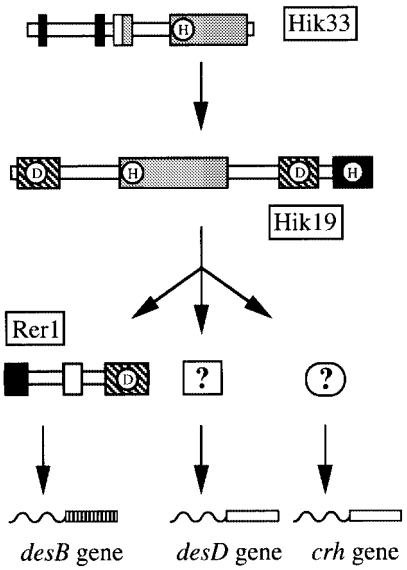

Fig. 6. A hypothetical scheme for the pathway for low-temperature signal transduction in *Synechocystis*. The histidine kinase domains, the receiver domains and the histidine phospho-transfer (Hpt) domain of Hik33, Hik19 and Rer1 are indicated by gray rectangles, hatched rectangles and a filled rectangle, respectively. The histidine and aspartate residues that might be involved in the phospho-relay reaction are indicated by H and D in circles, respectively. Filled rectangles, a gray rectangle and an open rectangle in Hik33 indicate the putative membrane-spanning domains, the coiled-coil domain and leucine-zipper domain, respectively. Closed and open rectangles in Rer1 indicate regions homologous to the HMG box and the Ah receptor nuclear translocator, respectively (see the text for details).

residues. The strongly conserved histidine kinase domain is located near the C-terminus. An analysis using computer programs that predict the localization of proteins, such as PSORT (Nakai and Horton, 1999) and HMMTOP (Tusnady and Simon, 1998), indicated that Hik33 has two hydrophobic helices that might, in theory, span the membrane. A putative leucine zipper motif and a putative coiled-coil sequence are located between the second hydrophobic helix and the histidine kinase domain. These motifs are involved in the dimerization of a number of histidine kinases and are important for their activities (Lau *et al*., 1997; Yaku and Mizuno, 1997; Singh *et al*., 1998). Thus, we can predict that one or both of these sequences might be involved in the dimerization of Hik33 and the regulation of its activity.

A reduction in the fluidity of the plasma membrane of a cyanobacterium appears to be a primary signal for the low-temperature-induced expression of the genes for desaturases (Vigh *et al*., 1993; Murata and Los, 1997). The properties of Hik33 appear to be consistent with those of a sensor that can detect a decrease in membrane fluidity. A reduction in the fluidity of the membrane at sites at which Hik33 is located might alter the structure of Hik33, influencing the spatial relationship between monomers of the dimerized protein and thereby altering activity.

We searched for proteins homologous to Hik33 in standard databases. The kinase domain of Hik33, which includes an autophosphorylatable histidine residue and an ATP-binding motif (Park *et al*., 1998), was very similar to those of histidine kinases from bacteria, yeast, fungi and plants. However, the sequences outside the kinase domain, including the membrane-spanning domains, appeared to be unique. One relatively homologous gene was identified, namely *ycf26*, which was found in the chloroplast genome of the red alga *Porphyra purpurea* (Reith and Munholland, 1995). This gene encodes a homolog of histidine kinase of 656 amino acid residues. About 48% of the residues outside the histidine kinase domain are identical to those in Hik33 (data not shown). The hydropathy profiles of Hik33 and Ycf26 are also very similar (data not shown). However, it is unclear what kinds of signal might be perceived and what genes might be regulated by Ycf26.

Hik33 is also homologous to a Ycf26-like protein found in the chloroplast genome of *Cyanidium caldarium* (DDBJ/EMBL/GenBank accession No. AF022186) and to a protein encoded by the *yycG* gene in *B.subtilis* (Kunst *et al*., 1997). These proteins also contain putative membrane-spanning domains and coiled-coil motifs. This indicates that a pathway for the perception and transduction of cold signals identified in *Synechocystis* might be a common feature of the responses of many organisms to cold.

Characteristics of Hik19

The amino acid sequence deduced from the *hik19* gene (sll1905) indicates that Hik19 contains 1014 amino acid residues and computer analysis suggests that it might be a soluble protein in the cytosol. A strongly conserved histidine kinase domain is located in a central region of the protein. One signal-receiver domain is localized at the N-terminus and another is near the C-terminal region. Furthermore, a histidine phospho-transfer (Hpt) domain is located at the C-terminus. Thus, Hik19 is a hybrid-type histidine kinase (Mizuno *et al*., 1996). The receiver domain at the N-terminus might accept a phosphate group from some other histidine kinase or protein that contains phosphorylated histidine and Hik19 might function downstream of the membrane-bound sensor Hik33. Hik19 is more likely than Hik33 to be a transducer of the low-temperature signal.

Characteristics of Rer1

The amino acid sequence deduced from the *rer1* gene indicates that Rer1 contains 402 amino acid residues. Unlike most response regulators that have a signal-receiver domain at the N-terminus, Rer1 has a signal-receiver domain at the C-terminus. However, the N-terminal region is homologous to the DNA-binding domain, known as an HMG box, found in regulators of transcription in vertebrates, such as the Sox6 and Sox5 proteins (Connor *et al*., 1995). The central part of Rer1 is similar to the transcriptional activation domain of the aryl hydrocarbon (Ah) receptor nuclear translocator (*Arnt*; Li *et al*., 1994; Figure 6). Although Rer1 does not have a structure typical of response regulators, it is possible that it functions as a DNA-binding regulator of transcription.

Characterization of the ΔHik33, ΔHik19 and ΔRer1 mutants

The extent of the low-temperature-inducible expression of the *desB* transcript in ΔHik33 cells was half that in wild-type cells (Figure 2). The low levels of Hik33 and Hik19 might be responsible for the low level of accumulation of the *desB* transcript in the mutant cells. However, we found that the low-temperature-induced activation of the *desB* promoter in *pdesB::lux*/ΔHik33 or *pdesB::lux*/ΔHik19 cells, monitored in terms of luciferase activity, was almost completely eliminated by the inactivation of the *hik19* genes or the *hik33* genes (Figure 1). The discrepancy between the extent of depression of the accumulation of the transcript and the extent of transcriptional activation might be explained by an increase in the stability of transcripts at low temperature, as shown in Figure 3 and in the previous report (Los *et al*., 1997). The half-life of the *desB* transcript increased 15-fold after a shift in growth temperature from 34 to 22°C in wild-type cells and in ΔHik33 and ΔHik19 cells. It is unclear how the transcripts of *desB*, *desD* and *desA* genes might be stabilized at low temperature.

Although inactivation of *hik33* and *hik19* reduced the low-temperature-induced accumulation of *desB* and *desD* transcripts (Figure 2), inactivation of the *rer1* gene resulted in a reduction in the level of the *desB* transcript, while levels of *desD*, *desA* and *crh* transcripts were unaffected (Figure 5). These results indicate that Hik33 and Hik19 might be involved in a common mechanism that regulates the expression of *desB*, *desD* and *crh* genes, and that Rer1 might specifically regulate the expression of the *desB* gene (Figure 6). In *E.coli*, a hybrid-type histidine kinase, ArcB, transfers a phosphate group via its Hpt domain to several receivers, such as ArcA, OmpR and CheY (Perraud *et al*., 1999). Hik19 might also transfer phosphate groups to some, as yet unidentified, response regulators that, perhaps, contain a receiver domain and it might, thus, regulate the expression of *desD*, *crh* and certain other low-temperature-inducible genes.

A hypothetical pathway for perception and transduction of low-temperature signals

Figure 6 shows a hypothetical scheme for the transduction of low-temperature signals. Hik33 may span the plasma membrane twice and forms a dimer, whose structure may be influenced by the physical characteristics of lipids in the plasma membrane, such as their fluidity (or the extent of molecular motion), which is controlled by temperature and the extent of unsaturation of the fatty acids. When the temperature is decreased or the fatty acids are more saturated, the histidine residue in the histidine kinase domain may be phosphorylated. A phosphate group is then transferred to Hik19, and finally to Rer1, which regulates the expression of the *desB* gene. Hik19 and Hik33 are also involved in the regulation of expression of the *crh* and *desD* genes. However, we have not yet identified the response regulators (or transcriptional regulators) of these genes.

In *E.coli*, heat stress induces the expression of several genes, whose products are involved in the folding and degradation of denatured proteins. Some of these genes are regulated by the typical two-component system CpxA–CpxR. CpxA is a histidine kinase, which is bound to the plasma membrane and is autophosphorylated under heat stress. Phosphorylated CpxA transfers a phosphate group to the response regulator CpxR, which activates the transcription of several heat-inducible genes, such as *degP*, which encodes a protease, and *dsbA*, which encodes a disulfide isomerase (Mileykovskaya and Dowhan, 1997). The polypeptide deduced from the *cpxA* gene is different from Hik33 except in its histidine kinase domain. The pathway for low-temperature signal transduction in *Synechocystis* (Figure 6) appears to be more complex than the two-component system for high-temperature signal transduction in *E.coli*.

The expression of several sets of genes in response to low temperature occurs in all poikilothermic organisms examined to date. A pathway for the perception and transduction of low-temperature signals that includes two histidine kinases identified in *Synechocystis* might be a common feature of the responses of many organisms to low temperatures.

Materials and methods

Cells and culture conditions

A strain of *Synechocystis* sp. PCC 6803, which is tolerant to glucose (Williams, 1988), was obtained from Dr Williams at Dupont Co. Ltd. We generated strain *pdesB::lux*, in which the coding region of the *desB* gene was replaced by the *luxAB* gene for bacterial luciferase, as described previously (Los *et al*., 1997). In this construct, the *luxAB* gene was expressed under the control of the *desB* promoter. Wild-type cells were grown at 34°C in BG-11 medium (Stanier *et al*., 1971) buffered with

20 mM HEPES–NaOH pH 7.5 under continuous illumination from incandescent lamps, as described previously (Wada and Murata, 1989). Cells of mutants in which the Sp^r cassette had been inserted into the genome were grown under the same conditions as described above with the exception that the culture medium contained spectinomycin at 20 μg/ml up to the pre-cultures. Mutant cells were then transferred to medium without spectinomycin for the final cell culture used for the experiments.

Systematic targeted mutagenesis of genes for histidine kinase

DNA fragments that contained complete or partial sequences of the 43 putative genes for histidine kinases of *Synechocystis* were amplified by PCR and cloned into pT7Blue (Novagen, Madison, WI), pBluescript II SK(+) (Stratagene, La Jolla, CA) or pUC18 (Toyobo, Osaka, Japan). Then the Sp^r cassette (Prentki *et al*., 1991) was inserted at suitable restriction sites in the Hik coding region in the same orientation as the open reading frame (Figure 1B). *pdesB::lux* cells were transformed with the resultant plasmids as described by Williams (1988). Transformed cells were cultured for at least 6 months on agar-solidified BG-11 medium (Stanier *et al*., 1971) that was buffered with 20 mM HEPES–NaOH pH 7.5 and contained 20 μg/ml spectinomycin. Genomic DNA was extracted from mutant cells and used as the template for analysis by PCR of the extent of replacement of copies of wild-type chromosomes by mutated chromosomes.

ΔHik33 and ΔHik19 were produced by inactivating the *hik33* and *hik19* genes in wild-type cells in the same way as described above for *pdesB::lux* cells, with the Sp^r cassette being inserted at the *Nco*I site in each gene.

Random mutagenesis by insertion of the Sp^r cassette and identification of genes responsible for activation of the desB promoter

We modified pUC18 to generate pUCBam, in which all the multiple cloning sites were eliminated except the *Bam*HI site. pUC18 was first treated with the restriction enzymes *Xba*I and *Eco*RI, and then sites of cleavage were blunted by T7 DNA polymerase (DNA Blunting Kit; Takara, Kyoto, Japan) and self-ligated. The resultant plasmid was digested with *Sal*I and *Hin*dIII, blunted and self-ligated to construct pUCBam. The genomic DNA extracted from wild-type cells of *Synechocystis* was partially digested with *Sau*3AI and fractionated by electrophoresis on an agarose gel. DNA fragments of 1–5 kb were recovered from the gel and inserted into the *Bam*HI site of pUCBam. The resultant genomic library was subjected to digestion with *Eco*RI, *Hin*dIII, *Xba*I, *Hin*cII, *Nco*I or *Nhe*I. The Sp^r cassette (Prentki *et al*., 1991) was ligated into the cleaved plasmids. *Escherichia coli* JM109 cells were transformed with the resultant plasmids. The transformed cells with Sp^r-tagged plasmids were selected on agar-solidified LB medium that contained 50 μg/ml ampicillin and 30 μg/ml spectinomycin. The Sp^r-tagged plasmids were recovered from selected *E.coli* cells and used for the transformation of *Synechocystis* cells as described by Williams (1988).

After the selection of mutants in which the low-temperature-induced activation of the *desB* promoter was depressed, we determined the DNA sequences on both sides of the sites of insertion of the Sp^r cassette in some of the mutants by inverse PCR (Ochman *et al*., 1988; Triglia *et al*., 1988) with DNA extracted from the mutant cells as template. Then we inactivated separately the genes located in the vicinity of the sites of insertion of the Sp^r cassette by targeted mutagenesis of *pdesB::lux* cells as described above. We identified genes responsible for the loss of inducibility by low temperature of the *desB* promoter by monitoring luminescence due to luciferase activity.

Determination of the activity of the desB promoter using a reporter gene for bacterial luciferase

Cells of *pdesB::lux* and mutants derived from it were grown on agar-solidified medium for 2 days at 34°C and then incubated at 22°C for appropriate periods of time. After *n*-decanal vapor had been applied to the cells for 1 min, the emission of photons from cells was monitored for 1 min with a photon-counting luminometer (ARGUS 50; Hamamatsu Photonics, Hamamatsu, Japan).

Analysis of transcripts

Northern blotting analysis was performed as described previously (Los *et al*., 1997). DNA fragments corresponding to the *desA*, *desB*, *desD* and *crh* genes were conjugated with alkaline phosphatase (Alkphos Direct kit; Amersham Pharmacia Biotech, Uppsala, Sweden) and the resultant conjugates were used as probes. After hybridization, the blots were soaked with CDP-star solution (Amersham Pharmacia Biotech) and signals from hybridized mRNA were detected with a luminescence image analyzer (LAS-1000; Fuji-Photo Film, Tokyo, Japan).

Acknowledgements

This work was supported by a Grant-in-Aid for Specially Promoted Research (No. 08102011) from the Ministry of Education, Science and Culture, Japan.

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Received November 16, 1999; revised and accepted January 21, 2000