A novel heat shock gene, orf7.5, which encodes a putative acidic polypeptide of 63 amino acids, was cloned from the cyanobacterium *Synechococcus* sp. PCC 7942. Northern blot analysis revealed the presence of 400- and 330-base orf7.5 mRNAs, which were barely detectable in the cells grown at 30 °C but increased transiently in response to heat shock at 40 or 45 °C. Primer extension analysis showed that the two mRNAs have different 5'-ends. Chloramphenicol enhanced the accumulation of the orf7.5 mRNA, whereas it inhibited the increase in the amount of the groESL mRNA. To reveal the role of the orf7.5 gene in thermal stress management, we constructed a stable mutant in which a gene conferring resistance to an antibiotic was inserted into the coding region of the orf7.5 gene. The interruption led to a marked inhibition of growth at 45 °C and a decrease in the basal and acquired thermo-tolerances at 50 °C in the transformants, indicating that the gene plays a role in thermal stress management. The orf7.5 mutant could be complemented with a return to the wild-type phenotype by a DNA fragment containing orf7.5 but not by mutated orf7.5-s, in which a nonsense mutation was generated by introducing a frameshift or a point mutation within the orf7.5-coding region. Thus, thermo-tolerance requires an appropriate translation product, not simply a transcript. Accumulation of the groESL transcript in the orf7.5 mutant was strongly reduced, suggesting that the orf7.5 gene product controls the expression of the groESL operon.

Cyanobacteria are photoautotrophic prokaryotes that are phylogenetically and physiologically related to the chloroplasts of photosynthetic eukaryotes. Thus, cyanobacteria, especially transformable ones such as *Synechocystis* sp. PCC 6803 and *Synechococcus* sp. PCC 7942, have proven to be valuable model organisms to elucidate chloroplast functions such as photosynthesis. Cyanobacteria, like other organisms, synthesize a diverse range of heat shock proteins (Hsps) upon exposure to high temperatures (1–4). To prove a specific contribution of a Hsp in thermo-tolerance in cyanobacteria, disruption mutants of the clpB, hsp16.6, and htpG genes have been constructed (5–7). Interestingly, all these disruptants showed much more striking thermo-sensitive phenotypes than those of *Escherichia coli* or other prokaryotes, suggesting that those Hsps have a particularly important role for photosynthetic prokaryotes. In fact, photosynthetic oxygen evolution activity in the clpB and hsp16.6 mutants was more susceptible to high temperature inactivation than that in the wild type (5, 6).

The regulation of the expression of cyanobacterial heat shock genes remains poorly understood. Webb et al. (3) identify sequences upstream of the transcription start site of the groESL gene from *Synechococcus* sp. PCC 7942 cells that were similar to the consensus heat shock promoters of *E. coli* recognized by sigma factor 32. However, there is no evidence for the presence of sigma factor 32 in cyanobacteria. As far as we know, there is no report that an alternative sigma factor may control the heat shock regulon in cyanobacteria. The nine-nucleotide inverted repeat sequence called CIRCE (8) is a regulatory element of heat shock induction that is conserved in cyanobacteria as well as other prokaryotes. The CIRCE element has been reported to be present around the transcription start site in groEL and/or dnaK operons of more than 30 different bacterial species, and it is thought to be an operator with which the HrcA repressor protein interacts (9–11). CIRCE has been identified upstream of groEL genes in cyanobacteria (12–14). Recently, it was shown that heat shock strongly enhanced accumulation of transcripts of the two groEL genes, groEL and cpn60, in *Synechocystis* PCC 6803 in the light, but induction was lower in the dark (14). Light appears to exert its effect through the photosynthetic electron transport since DCMU, an inhibitor of electron transport, suppressed the accumulation of transcripts of the two groEL genes in the light. Those results suggest that cyanobacteria may have evolved a unique regulatory mechanism to induce the groEL genes. Evidence for additional regulatory mechanisms came from an inspection of promoter sequences. The potential transcription initiation site of the hspA gene, encoding a small Hsp homologue in the thermophilic cyanobacterium *Synechococcus vulcanus*, was preceded by typical vegetative promoter sequences, although its transcript was clearly heat-inducible (15). There was no CIRCE element around the potential transcription initiation site of the hspA gene, indicating that an unknown regulatory mechanism suppresses the expression of hspA in cyanobacteria under non-heat shock conditions. Furthermore, the gene appears to be regulated post-transcriptionally because its mRNA was more stable at a heat shock temperature 63 °C than at 50 °C (15).

In this paper, we report the isolation and characterization of...
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a novel heat shock gene, orf7.5, from Synechococcus sp. PCC 7942. To reveal the function of the orf7.5 gene, we generated a mutant strain in which orf7.5 was insertionally disrupted by gene targeting. Analysis of the mutant showed the involvement of the orf7.5 gene in the growth and survival of Synechococcus sp. PCC 7942 under high temperatures and, additionally, in the expression of the groESL operon.

**EXPERIMENTAL PROCEDURES**

**Organisms and Culture Conditions**—Unless otherwise indicated, Synechococcus sp. PCC 7942 cells were grown in BG-11 medium supplemented with riboflavin, thiamine, and biotin, pH 10, and incubated in 150 µmol m⁻² s⁻¹ at 30 °C under continuous illumination. The cell density was 5 x 10⁶ cells ml⁻¹. The culture was bubbled with air, and the liquid culture was washed in 1.5% (w/v) agar and 0.3% (w/v) sodium thiosulfate. The culture was subcultured every 3 days.

**DNA Sequencing**—Sequencing single-stranded DNA was carried out by using an AutoRead sequencing kit (Amersham Pharmacia Biotech) and a DNA sequencer (DSQ-1, Shimadzu, Kyoto, Japan). The nucleotide sequences were aligned and analyzed using GENETYX software (Software Development Co., Ltd., Tokyo, Japan).

**Probe Preparation and Genomic Library Screening**—The orf7.5 gene from Synechococcus sp. PCC 7942 was found during the amplification of a small Hsp gene by polymerase chain reaction (PCR). One of the PCR primers was designed using the amino-terminal sequence of the HspA of *S. vulcanus* (15), EPFREED, and the other primer, 5'-ATIC/TA/GA/IGTIA/GAIACCC-3', was based on the internal amino acid sequence of the Hsp18 of *Synechocystis* algae (17), GVTLTR. A 300-bp fragment was amplified from the Synechococcus sp. PCC 7942 genome after 90 cycles of denaturation for 1 min at 94 °C, annealing for 1 min at 55 °C and extension for 2 min at 72 °C, although nothing was produced after 30 PCR cycles. The product was designated as PCR-1. The PCR-1 was cloned into pT7Blue T-vector (Novagen, Madison, WI). PCR-1 was labeled with [α-³²P]dCTP by the multiprime-labeling method as directed by the manufacturer (Amersham Pharmacia Biotech). The probe was used to screen a Synechococcus sp. PCC 7942 genomic library constructed in bacteriophage λ-DASH vector. Bacteriophage DNA from positive plaques was prepared by the liquid culture method, and further screening was performed by Southern blot analysis (18) after digestion of the DNA by EcoRI. A 6.5-kb fragment that hybridized with the above probe was subcloned into plBluescript II KS (+) (Stratagene, La Jolla, CA). A 5.0-kb XhoI-EcoRI fragment was generated by digestion of the 6.5-kb EcoRI fragment with XhoI and was subcloned into pBluescript II KS (+). The 5.0-kb XhoI-EcoRI fragment was obtained by the Erase-a-Base System (Promega, Madison, WI). With M13KO7 as a helper phage, single-stranded templates from both strands were generated for sequencing.

**Isolation of Genomic DNA from Synechococcus sp. PCC 7942 and Southern Blot Analysis**—Southern blot analysis was performed as described previously (19) except for the following procedure. A 276-bp XhoI-EcoRI fragment (Fig. 1A) containing the 5.0-kb XhoI-EcoRI fragment was obtained by the Erase-a-Base System (Promega, Madison, WI). With M13KO7 as a helper phage, single-stranded templates from both strands were generated for sequencing.

**Preparation of Total RNA from Synechococcus sp. PCC 7942 and Northern Blot Analysis**—Total RNA from Synechococcus sp. PCC 7942 was prepared as described previously (19) from cells incubated for different time intervals after shifting cultures from 30 to 40 or 45 °C. During the incubation, the culture was illuminated and air-bubbled as described above. Unless otherwise indicated, 10 μg of total RNA was electrophoresed on a denaturing 1.5% (w/v) agarose gel containing 6.6% (w/v) formaldehyde. Northern blotting and hybridization with the [³²P]-labeled 276-bp XhoI fragment (Fig. 1A) as a probe were performed as described previously (19). After hybridization, the membrane was washed in 6x SSC at 65 °C for 60 min. The size of the mRNA was determined using an RNA ladder (Life Technologies, Inc.). The hybridization signals were detected with a BAS1000 Mac bio-imaging analyzer (Fuji Film, Tokyo, Japan).

**Reverse Transcription-PCR**—Reverse transcription-PCR was performed with one gene, groES, using the GeneAmp thermostable Taq reverse transcriptase PCR kit as directed by the manufacturer (Roche Molecular Biochemicals). Total RNA was prepared as described above, and contaminating DNA was removed by S.N.A.P. total RNA isolation kit (Invitrogen, Carlsbad, CA). The antisense oligonucleotide primer (5'-TATTTCTTACG-GCTTTGGCCGA-3') complementary to nucleotides 693–712 (Fig. 1) was used for cDNA synthesis that was performed at 60 °C for 30 min. The resulting cDNA was amplified using GeneAmp thermostable rTth reverse transcriptase RNA polymerase and dideoxynucleotide sequencing reactions performed with the same primer and the cloned 5.0-kb XhoI-EcoRI fragment as a template were run in parallel to allow determination of the end points of the extension products.

**Construction of a Mutant**—A 2.0-kb HindIII/EcoRI chromosomal fragment (Fig. 1A) cloned in plBluescript II KS (+) was digested at the unique BstEII site in orf7.5. A kanamycin-resistant gene cassette, which was isolated by digesting pUC4K (Amersham Pharmacia Biotech) by BamHI, was inserted into the restriction site in the forward direction (the same gene orientation as that of orf7.5). The resulting plasmid was named pTS31 (Fig. 5). The construct was used to transform cells of naturally competent *Synechococcus* sp. PCC 7942 through homologous recombination. These cells were segregated for a few generations by single colony selection on BG-11 agar plates to isolate mutant strains (20). 20 µg/ml kanamycin sulfate was used for selection of transformants. One of the clones was designated as NT31.

**Viability Assays**—The *Synechococcus* sp. PCC 7942 cells were grown to log phase at 30 °C under a light intensity of 35 µE m⁻² s⁻¹ as described above and then harvested in an absorbance of 0.5 at 730 nm. For basal thermo-tolerance assays, 20 ml of the diluted culture was incubated at 50 °C for 20 min. Aliquots of the culture taken before and after the high temperature treatment were serially diluted (5 times each) in fresh, sterile BG-11 medium. A 10-µl aliquot from each dilution was then spotted onto a BG-11 plate, and the culture was grown at 30 °C under a light intensity of 35 µE m⁻² s⁻¹ for a week. Cell survival was determined using a colony-counting method. The survival rate of the wild type was compared to that of the mutant at each time point.
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Site-directed Mutagenesis of the orf7.5 Gene—A frameshift within the orf7.5-coding region was generated by QuikChange site-directed mutagenesis kit (Stratagene). Two primers (5'-GGTTCGTTACCTCTGACGAAAATCTTGGGGC-3', 5'-CCCAAGATTTGCTGAGGATTCGACAACCG-3') containing an insertion of a single base at the ninth codon of orf7.5 (underlined bases in the above sequences, see also Fig. 8A), each complementary to opposite strands of the vector pBS-EP, were extended by PCR. Then the product was treated with DpnI to digest the parental template pBS-EP and to select for mutation-containing synthesized DNA. The DNA incorporating a frameshift in orf7.5 was designated as pNI71. In the same way, pNI81 in which a point mutation was incorporated at the third codon of orf7.5 (underlined bases in the following sequences, see also Fig. 8A), was generated using two primers, 5'-CACTCTACATGCGTGTTACCTCTGGTACCG-3' (underlined bases in the following sequences), 5'-CCGACGGTTACCTCATGAGGATTCGACACCG-3'. The mutations were confirmed by sequencing orf7.5 in pNI71 and pNI81. Either pNI71 or pNI81 was introduced into Synechococcus sp. PCC 7942 as described above, and transformants were selected on BG-11 agar plates containing 10 μg/ml kanamycin and 7.5 μg/ml chloramphenicol. One of the transformants was designated as N152.

Cloning and Sequencing of Two Novel Open Reading Frames and purB from Synechococcus sp. PCC 7942—Initially, we intended to amplify a small Hsp gene from the unicellular cyanobacterium Synechococcus sp. PCC 7942 by PCR with degenerate primers to small Hsps from other prokaryotes. Although there was no product after 30 cycles of polymerase reactions, further-extended PCR resulted in a 0.3-kbp DNA fragment. The sequence of the DNA fragment does not reveal close relatedness to any gene in the data base. We further examined the product to see if it is a portion of a heat-inducible gene since we have embarked on a project to identify cyanobacterial Hsps and their genes (7, 13, 15, 21). Northern blot analysis revealed transient increases during heat shock of 400- and 330-base RNAs with which the 0.3-kbp PCR product hybridized (data not shown). These results indicated that the PCR product constitutes a novel heat shock gene. Thus, we went on to clone a DNA fragment containing the whole gene by screening a genomic library (see Fig. 1) will appear in the DDBJ, EMBL, and GenBank databases.

RESULTS

Cloning and Sequencing of Two Novel Open Reading Frames and purB from Synechococcus sp. PCC 7942—Initially, we intended to amplify a small Hsp gene from the unicellular cyanobacterium Synechococcus sp. PCC 7942 by PCR with degenerate primers to small Hsps from other prokaryotes. Although there was no product after 30 cycles of polymerase reactions, further-extended PCR resulted in a 0.3-kbp DNA fragment. The sequence of the DNA fragment does not reveal close relatedness to any gene in the data base. We further examined the product to see if it is a portion of a heat-inducible gene since we have embarked on a project to identify cyanobacterial Hsps and their genes (7, 13, 15, 21). Northern blot analysis revealed transient increases during heat shock of 400- and 330-base RNAs with which the 0.3-kbp PCR product hybridized (data not shown). These results indicated that the PCR product constitutes a novel heat shock gene. Thus, we went on to clone a DNA fragment containing the whole gene by screening a genomic library (see Fig. 1). One of them, designated as orf14.1 (the number is based on the estimated molecular weight for the orf) is located from bases 333 to 713 of the sequence shown in Fig. 1B. Within orf14.1, there was another orf that we designated as orf7.5. The nucleotide sequence of the region that contains orf14.1, orf7.5, and the 5'-end of purB. Nucleotides are numbered from the 5'-end, and the deduced amino acid sequences of orf14.1, orf7.5, and purB are shown below the corresponding DNA sequences. The deduced amino acid sequence of orf7.5 is shaded. Putative 10 and 35 sequences are underlined. The putative Shine-Dalgarno sequence is double-underlined. Putative transcription start sites (P1 and P2) determined by primer extension analysis, are indicated with vertical arrows. A sequence of dyad symmetry downstream of orf7.5, which could act as a transcriptional terminator, is indicated with arrows.

One of them, designated as orf14.1 (the number is based on the estimated molecular weight for the orf) is located from bases 333 to 713 of the sequence shown in Fig. 1B. Within orf14.1, there was another orf that we designated as orf7.5. The nucleotide sequence of the region corresponding to a region from bases 478 to 607 of the sequence shown in Fig. 1B. The latter half of the PCR product did not correspond to the sequence. Thus, the 400- and 330-base RNAs described above are likely to be transcripts of orf14.1 and/or orf7.5. The amino acid sequence deduced from the larger orf, which is located 54-bp downstream of orf14.1, showed significant homology to adenylosuccinate lyase (purB) of Bacillus subtilis (22).

The number of orf14.1 and orf7.5 copies in the Synechococcus...
Heat Induction of orf14.1 and/or orf7.5—To confirm that orf14.1 and/or orf7.5 is induced by heat shock, we performed Northern blot analysis with total RNA isolated from cells after a shift from 30 to 45 °C. The NcoI DNA fragment containing a 3′-portion of orf14.1, whole orf7.5, and upstream untranslated region of the purB gene hybridized with 400- and 330-base RNAs (Fig. 2A). The 400- and 330-base RNAs, barely detectable at 30 °C, increased 4- and 3-fold, respectively, after a 15-min exposure to 40 °C and then diminished during 45 min of prolonged incubation (Fig. 2A). The Northern blot was stripped and reprobed with the 32P-labeled orf7.5 gene of Synechocystis sp. PCC 6803 (23) (C).

The DNA and RNA fragments used as probes contain sequences preceding P2. P1 was located 70 bases upstream of orf14.1 and orf7.5 (data not shown).

The 400- and 330-base RNAs were the transcripts of orf14.1 and/or orf7.5.

The 400- and 330-base mRNAs Were the Transcripts of orf7.5—The two RNA species of 400 and 330 bases may be transcripts of orf14.1 and orf7.5, respectively. Alternatively, both mRNAs originate from the same orf. To clarify the origin of the RNAs, we determined the 5′-ends of the 400- and 330-base mRNAs by primer extension analysis using total RNA and a synthetic oligonucleotide primer complementary to the 5′-end of orf7.5. Total RNA was isolated from Synechococcus sp. PCC 7942 cells grown at 30 °C or cells heat-shocked at 45 °C for 15, 30, 60, and 120 min. The analysis revealed two extended products (Fig. 3). No more extended products were detected within 200 bases upstream of P1 (data not shown). The result indicated that the two mRNAs have different 5′-ends that correspond to the P1 and P2 positions of the genomic DNA shown in Fig. 1B. The number of both transcripts increased with the temperature shift from 30 to 45 °C, although the transcripts initiated at P1 increased much more than those initiated at P2. P1 and P2 were located 141 and 71 bases upstream of the ATG start codon of orf7.5, respectively (Fig. 1B and Fig. 3). Since P1 and P2 are located within orf14.1 (Fig. 1A), the two mRNAs are likely to be the transcripts of orf7.5. We could find neither the E. coli-type heat shock promoter sequences recognized by sigma factor 32 (25) nor the CIRCE (8) sequence upstream of P1 and P2. Instead, P1 was preceded by sequences that resemble the consensus sequences of promoters recognized by the vegetative sigma factors of B. subtilis as well as that of E. coli (Fig. 1B, underlined) (25). We could not find any promoter sequences preceding P2. P1 was located 70 bases upstream of P2 (Fig. 1B), supporting the conclusion that the 400- and 330-base RNAs (Fig. 2B) have the same 3′-end.

We confirmed by reverse transcription-PCR that transcription of orf14.1 must be negligible even if it is actually ex-
pressed. There was only a negligible, if any, amount of PCR products with the expected size (0.43 kbp) when a primer corresponding to the sequence 41-bp upstream of P1 and a primer complementary to the 3' end of orf14.1 were used for the PCR reaction to amplify a DNA fragment complementary to the orf14.1 mRNA (data not shown). In addition, we constructed a mutant in which an antibiotic resistance gene was inserted into a site located 38 bases upstream of the first ATG codon of orf14.1. The insertion did not cause any apparent phenotype at both 30 and 45 °C. Thus, we conclude that orf14.1 does not function as an expressed gene.

Differential Effect of Chloramphenicol on the Expression of the groESL Operon and orf7.5—There is no CIRCE regulatory element around the orf7.5 transcription start site, whereas the groESL operon has one. Thus, we thought that there should be some difference in the heat induction of the two genes. Previously, we showed the protein synthesis inhibitor chloramphenicol completely inhibited the accumulation of mRNA from the groESL operon in S. vulcanus when added before the heat shock (13). This suggested the involvement of heat-induced production of a protein for the expression of the operon. We attempted to determine whether the antibiotic may exert a different effect on the expression of the orf7.5 gene. When chloramphenicol was added before the shift from 30 to 45 °C, the accumulation of the 400- and 330-base RNAs continued during the heat shock (Fig. 4A). The increase in the level of mRNA was remarkable. These results were in contrast with the transient increase of the mRNA accumulation in the control (Fig. 4A). The Northern blot was stripped and reprobed with a 32P-labeled groEL gene from S. vulcanus (13). As opposed to the increased accumulation of the 400- and 330-base RNAs in the control, that of the groESL mRNA was completely inhibited by the addition of chloramphenicol (Fig. 4B).

Disruption of orf7.5—To elucidate the function of the orf7.5 gene in relation to thermo-tolerance, the gene was disrupted by inserting a kanamycin-resistant gene cassette into the BstEII site located 19 bp downstream of the initiation codon of orf7.5. The mutant was designated as NT31 (Fig. 5).

Integration and complete segregation of the orf7.5 interruption construct within the mutant genome was confirmed by Southern blot analysis and PCR analysis (data not shown). The size and the number of the restricted DNA fragments hybridized with the probe and the PCR-amplified products were as expected when the kanamycin-resistant gene cassette (1.3 kbp) was introduced into the loci shown in Fig. 5 in all the copies of the mutant chromosome. 400- and 330-base RNAs were not detected in the mutant cells (Fig. 9). These results indicate that orf7.5 is dispensable for the cyanobacterial growth under the normal conditions.

Role of orf7.5 in Thermo-tolerance—We examined whether the interruption of orf7.5 has any effect on the thermo-tolerance of Synechococcus sp. PCC 7942. The growth rates of the wild type and the mutant, NT31, were measured at 30 or 45 °C by monitoring the apparent absorbance of the cultures at 730 nm. The growth rate of the mutant was similar to that of the wild type at 30 °C (Fig. 6). However, when the temperature was increased to 45 °C, the mutant NT31 stopped growing after a short initial growth period of ~20 h (Fig. 6), which almost equals the time required for one cell division. The wild type could grow at this temperature. The temperature-sensitive phenotype of NT31 showed that orf7.5 plays an important role in cyanobacterial growth even at moderately high temperature.

We attempted to determine whether there is any difference in basal thermo-tolerance between the wild type and the mutant NT31. Basal thermo-tolerance was determined as the percentage of cells surviving after a direct shift of cultures from 30 °C to a lethal temperature of 50 °C. Cells were incubated at 50 °C for 20 min in the light and cultured on a plate at 30 °C for a week, and then the number of colonies was counted. As shown in Fig. 7A, the survival rate of NT31 (0.2%) was 2 orders of magnitude lower than that of the wild type (49%), indicating that the expression of orf7.5 plays an important role in basal thermo-tolerance.

Acquired thermo-tolerance was determined as the percentage of cells surviving after exposure to 50 °C following a 60-min pretreatment at 42 °C (Fig. 7B). 42 °C is high enough to induce at least the htpG gene and the groESL operon (data not shown). The pretreatment gave full protection against the lethal temperature to the wild type. Although the mutant NT31 also acquired a remarkable thermo-tolerance through pretreatment, the survival rate of the mutant was half that of the wild type.

Fig. 4. The effect of chloramphenicol on the accumulation of orf7.5 mRNA (A) or groESL mRNA (B) in Synechococcus sp. PCC 7942. Cells were grown at 30 °C (lane 1) and then divided into two portions. To a 200-ml culture, 2.0 ml of 100% ethanol was added, and the culture was incubated at 30 °C for 5 min (lane 2, 0 min). Then, the culture was shifted to 45 °C for 15, 30 and 60 min (lanes 3–5, respectively) (shown by closed circles). To another 200-ml culture, 2.0 ml of chloramphenicol (30 mg/ml in 100% ethanol) was added, and the culture was incubated at 30 °C for 5 min (lane 6). Then the culture was shifted to 45 °C for 15, 30, and 60 min (lanes 7–9, respectively) (shown by open circles). Northern blot analysis with total RNA (10 μg of nucleic acid) obtained at various times was carried out with the radiolabeled Ncol fragment, and the combined levels of the 400- and 330-base orf7.5 mRNAs were quantitated with a Bio-imaging analyzer (BAS1000 Mac-BAS, Fuji Photo Film). After the analysis of the orf7.5 mRNA, the blot was stripped as described in Fig. 2. The membrane was reprobed with a radiolabeled 540-bp PCR product containing a part of the groEL1 gene of S. vulcanus (13), and the transcript levels were quantitated as for the orf7.5 mRNA.
Complementation of NT31 with a DNA Fragment Containing orf7.5—The 0.8-kbp DNA fragment containing orf7.5 (Fig. 1A) was introduced into a neutral site of the NT31 chromosome, resulting in NI52. Both the growth of NI52 at 30 and 45 °C under the light intensity of 30 µE/m²/s and the color of the NI52 cultures were indistinguishable from those of the wild type (Fig. 8B and data not shown). On the other hand, growth of NT31 ceased after 1 day at 45 °C (Fig. 8B), and the color of the culture became white and transparent after a few days at that temperature (data not shown). The DNA fragment contains only the first 16 codons of the purB-coding region (Fig. 1B). Thus, this result excludes the possibility that the thermolability of NT31 is due to a polar effect on the expression of the purB gene (located downstream of orf7.5) caused by the insertion of an antibiotic gene in orf7.5.

The Translation of orf7.5 Is Essential for the Thermotolerance—To test the hypothesis that the Orf7.5 protein is involved in thermo-tolerance, we decided to introduce either a frameshift or a point mutation within the orf7.5-coding region and attempted to determine whether these mutated orf7.5 genes can complement NT31, returning it to the wild type phenotype. Fig. 8A presents the DNA sequence of the 5'-region of orf7.5 and, below it, the sequences of the two mutated orf7.5 genes introduced into the neutral sites of the NI71 and NI81 chromosomes. In both NI71 and NI81, a nonsense mutation was generated by either inserting or changing a single nucleotide. Neither mutant could complement NT31, returning it to the wild type phenotype when mutant growth at 45 °C was compared with the wild type (Fig. 8C). Thus, the translation product of orf7.5 appears to be essential for the thermo-tolerance of Synechococcus sp. PCC 7942.

A typical Shine-Dalgarno sequence (AGGAGGA) (26) was found upstream of the first ATG of orf7.5 (Fig. 1B). In fact, the putative sequence was recognized by E. coli ribosomes. We subcloned the 2.0-kbp HindIII-EcoRI fragment (Fig. 1A) containing the whole sequence of orf7.5 and 131-bp upstream sequence of its start codon into pBluescript II KS (+). An E. coli strain harboring the plasmid produced large amounts of a 7.5-kDa protein when 1 mM isopropylthio-β-D-galactoside was added to the culture (data not shown). The amino-terminal sequence and the molecular mass of the recombinant...
protein revealed that it was the translation product of orf7.5 (data not shown).

Possible Regulation of the Expression of the groESL Operon and htpG Gene by orf7.5—We attempted to determine whether the interruption of orf7.5 may have some effect on expression of other heat shock genes, thus causing the temperature-sensitive phenotype described above. The accumulation of a major transcript of 2.3 kb that hybridized with a labeled RNA complementary to the bicistronic groESL mRNA was enhanced in the wild type cells by heat shock at 45 °C (Fig. 9). However, it was strongly inhibited in NT31. An RNA probe complementary to the htpG mRNA hybridized with a major transcript of 1.5 kb and a minor one of 2.2 kb. These mRNAs in the wild type cells increased in response to the heat shock. The smaller mRNA may be a degradation or processed product of the 2.2-kb htpG mRNA. Independent replicate experiments utilizing different cultures also resulted in the major transcript of 1.5 kb. The accumulation of those mRNAs was only slightly inhibited in NT31 (Fig. 9).

**DISCUSSION**

This report presents a novel heat shock gene, orf7.5, cloned from a mesophilic Synechococcus sp. PCC 7942. The gene, orf7.5, encodes a putative polypeptide of 63 amino acids with a predicted molecular mass of 7,455 Da and a pI of 4.97. We could not find any nucleotide or amino acid sequences in the data bases (GenBank™, EMBL, Swissprot or Cyanobase) that exhibit significant homology to this orf. The reason for the absence of a homologous sequence in data bases may be that orf7.5 is a small orf and located within a larger orf.

Northern blot and primer extension analyses revealed two transcripts of orf7.5 that have different 5’-ends (Figs. 2 and 3). The results suggest that either orf7.5 has two transcription start sites or the smaller transcript is a degradation product of the larger one. The absence of any obvious promoter sequences preceding P2 suggests that the 330-base mRNA is a degradation product of the 400-base mRNA. However, we cannot eliminate the possibility of the presence of an unknown promoter. If this is the case, the apparent difference in the amount of the two transcripts transiently accumulated upon heat shock (Figs. 2 and 3) may be due to the involvement of different promoters for the two transcripts.

Despite being transcribed from an apparent vegetative sigma factor-dependent promoter sequence, the transcript of orf7.5 was heat-inducible. Our results suggest that an unknown regulatory mechanism suppresses the expression of orf7.5 in cyanobacteria under non-heat shock conditions. The upstream region of orf7.5 contains no CIRCE element, indicating that the heat induction mechanism for the orf7.5 gene must be different from that for the groESL operon. The regulatory difference between the two genes is evident in the differential effect of chloramphenicol. The accumulation of groESL mRNA was completely inhibited by chloramphenicol added before heat shock, whereas that of orf7.5 mRNA was promoted remarkably (Fig. 4). Thus, heat-induced de novo synthesis of a protein(s) may be necessary for heat induction of the groESL operon, whereas it may repress that of the orf7.5 gene. As shown in Fig. 9, the inactivation of orf7.5 abolished the heat-induced accumulation of the groESL mRNA. Furthermore, it is the translation product of orf7.5 that exerts the thermo-resistance (Fig. 8). An attractive hypothesis is that the Orf7.5 protein acts as a positive regulator for heat induction of the groESL operon.
whereas it is a negative regulator for the orf7.5 induction. Thus, the orf7.5 gene may be self-regulated in its transcription.

The photoautotrophic growth of the orf7.5 mutant, NT31, was strongly inhibited at 45 °C (Fig. 6). The mutant was much less viable than the wild type at 50 °C (Fig. 7A). The thermostability phenotype of the mutant indicates that orf7.5 plays a role in the growth and survival of Synechococcus sp. PCC 7942 at high temperatures. The ability of the mutant to gain tolerance to short exposures at 50 °C by pre-exposure to 42 °C was also reduced (Fig. 7B). The thermostability phenotype of the mutant was strongly inhibited at 45 °C (Fig. 6). The mutant was much less viable than the wild type at 50 °C (Fig. 7A). This is because a 0.8-kbp fragment containing orf7.5 could complement NT31 (Fig. 8B). Furthermore, the same fragment containing orf7.5 with a nonsense mutation could not complement NT31 back to the wild type phenotype (Fig. 8C). These results strongly support the idea that the Orf7.5 protein, but not RNA, is involved in the thermostolerance of Synechococcus sp. PCC 7942. The high temperature-sensitive phenotype of NT31 may be due to the reduced expression of the groESL operon (Figs. 9).

Our present results suggest that the novel heat shock protein encoded by orf7.5 is involved in the heat induction of the groESL operon and plays an important role in thermal stress management in cyanobacteria. We postulate that the translation product of orf7.5 alone or together with other proteins interacts with the groESL operon at the DNA level and/or RNA level to regulate their heat induction.

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