ランソウの熱ショックタンパク質の発現調節とシャペロン複合体の解析

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はしがき

細胞の熱ショック応答の特徴は、高温依存的に幾種もの異なる熱ショックタンパク質（Hsp）遺伝子の転写産物が一過的に急激に増加し、高温下の翻訳がHsp遺伝子選択的に起こることである。細胞に蓄積したHspは分子シャペロンとして変性タンパク質の不可逆的凝集阻止や天然の構造への再生、あるいはプロテアーゼとして変性タンパク質の分解に働いていると考えられている。我々は既に、HtpGや低分子量Hspがランソウの高温耐性や光合成機能（集光・電子伝達活性）の熱耐性獲得等に重要な役割を果たしていることを明らかにしてきた。本研究では、光合成独立栄養生物のモデル生物としてランソウを実験材料に用い、Hsp遺伝子発現調節機構とその選択的翻訳機構の解明、光合成独立栄養生物特有のHsp遺伝子発現調節機構の解明、GroELやHtpGの細胞機能の解明、及びHtpGを含む複数種のHspから構成される複合体の単離と構造・機能解析を行なうことを目的とした。

Hsp遺伝子発現調節機構に関する最も顕著な研究成果は、我々が *Synechococcus* sp. PCC 7942 からその遺伝子をクローニングした新規なHsp（Orf7.5）が相互作用することにより、主要シグマ因子（RpoD1）が *groEL* 遺伝子の5'上流調節領域と結合するようになったことである。シグマ因子はRNAポリメラーゼのコア酵素と結合して初めてDNA結合能を獲得すると考えられていることから、コア酵素に依存しないシグマ因子のプロモーター領域への結合は極めて新規な発見である。Orf7.5がシグマ因子と相互作用し *groESL* オペロンの転写を正に調節するのではないかとの作業仮説をたて、*in vitro* の転写系でOrf7.5がRpoD1を結合したRNAポリメラーゼの転写活性を調節するかどうかを調べている。

groEL 遺伝子の発現調節に関しては、大腸菌などで詳細に解析されているストレス特異的シグマ因子（σ^{32}）が関与する正の調節と、枯草菌などにおけるCIRCE配列へのHrcAリプレッサーの結合による負の調節機構が知られている。*Synechocystis* sp. PCC 6803の二種類の *groEL* 遺伝子の転写開始点近傍にはCIRCE配列が存在しHrcAホモログをコードする遺伝子もゲノム上に存在することから、これらのHsp遺伝子の転写調節はCIRCE/HrcA抑制機構によるものと推察された。これを実証するために、*Synechocystis* sp. PCC 6803の *hrcA* 遺伝子破壊株を構築した。通常生育条件下における *groEL* 遺伝子の転写抑制が解除されたが、変異株の *groEL* 遺伝子のmRNA蓄積量が熱ショックにより、さらに著しく増加したので、CIRCE/HrcA抑制機構以外にも転写調節機構が存在することが示唆された。変異株の *groESL* オペロンの転写開始点は、熱ショック前後で同一であり、その上流には典型的な主要シグマ因子に認識されるプロモーター配列が存在したことから、主要シグマ因子を結合したRNAポリメラーゼ活性が熱ショックで増加するのではないかと考えた。Orf7.5のような新規なHspが、熱ショック条件下で主要シグマ因子を介してRNAポリメラーゼの活性調節に関与するという新しい作業仮説に基づき研究を進めている。

ランソウHsp遺伝子発現調節に関与するシス作用領域としては、上に述べたCIRCE以外には報告がなかった。本研究において、好熱性ランソウ *Synechococcus vulcanus* 低分子量Hsp遺伝子（*hspA*）の転写開始点と翻訳開始点の間に位置する新規なシス作用領域を同定した。この領域は、AとTが多く含まれる不完全なinverted repeat (ACAAGCAAATTTAGTTGT)配列をもつが、この配列に特異的に結合するタンパク質をゲルシフト法により検出した。*In vitro* では、この配列を含むDNA断片とDNA結合タンパク質は、50℃においては比較的安定な複合体を形成したが、熱ショック温度（63℃）では形成しなかった。細胞を63℃に移行後すぐにこのタンパク質のDNA結合活性は、

一過的に著しく減少したが、熱ショックに伴うこのタンパク質の生合成と安定化のために、DNA結合活性は50℃のレベルまで回復した。DNA結合活性の経時変化と*hspA*のmRNAの蓄積量の変化には負の相関があった。このDNA結合タンパク質を*hspA*発現を抑制する新規なリプレッサーと仮定して、現在その遺伝子のクローニングを行っている。

Hsp遺伝子の転写後レベルの調節機構に関する報告は全く無いが、我々は、*hspA*のコード領域に存在するdownstream box様配列とリボソームとの相互作用により高温における*hspA*転写産物の安定化や翻訳効率の増加が誘導されることを示唆する結果を得た。これが*hspA*遺伝子の選択的翻訳に関与するのではないかと考えて研究を進めている。

生物に普遍的に観察される熱ショック応答、即ち、ストレス後迅速に一過的に起こる転写発現が、ランソウでは光によって制御されることを示し、光が独立栄養生物の熱ショック応答に重要な役割を果たすことを初めて明らかにした。

GroEL やHtpGの細胞機能に関する以下のような新しい知見を得た。上述のように我々は*hrcA*遺伝子破壊株を構築したが、この変異株はGroELを構成的に発現した。遺伝子破壊株の高温（48℃）における生存率や光合成の集光機能（フィコシアニン）の熱安定性が増加した。また、HtpGは高温のみならず、低温や強光下でもランソウの生育・生存に重要であることを明らかにした。弱光下でも*htpG*遺伝子破壊株はメチルビオローゲンに感受性であることから、HtpGは酸化ストレス条件下で重要な機能を果たすのではないかと考えて、細胞内標的タンパク質の探索を行なっている。

GroEL及びDnaKと、48 kDaの未知のタンパク質を含む450 kDaの新規な複合体が低温で誘導されることを発見した。この複合体の蓄積は*htpG*遺伝子破壊株では阻害されることから、HtpGがその形成に関与すること、さらにこの複合体形成が低温順化に重要であることが示唆された。現在、GroELやDnaKタンパクを精製して、*in vitro*におけるマルチシャペロン複合体の再構築を試みている。大腸菌のGroELが安定な14量体をつくるのに対して、ランソウのGroELは不安定で、容易に単量体や二量体に解離した。このようなGroELの特性が他のHspとの複合体形成に重要ではないかと考えている。

研究組織

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≪ミニノート≫

シアノバクテリアの熱ショック応答

Heat shock response in cyanobacteria

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Heat shock proteins are families of proteins which display an enhanced expression in response to heat shock or other stresses. Heat shock proteins are ubiquitous, and their primary sequences are highly conserved among different species. Many heat shock proteins are molecular chaperones and play essential roles in protein folding, repair, translocation, and degradation.

Cyanobacteria are photoautotrophic prokaryotes whose habitats are extensive, indicating that they have adapted to various environmental conditions. Heat shock proteins may well have been playing important roles in their successful adaptation.

We cloned genes encoding HtpG and small HSP from cyanobacteria. Inactivation of the *htpG* gene resulted in a decrease in the cell's ability to develop thermo-tolerance (i.e., acquired thermo-tolerance), while over-production of small HSP increased the basal thermo-tolerance which is estimated by cell survival after a sudden lethal temperature treatment. In contrast to cyanobacteria, heterotrophic prokaryotes such as *E. coli* and *B. subtilis*, phenotypes of the *htpG* and small HSP mutants are not clear. Recently, we cloned a novel heat shock gene, *orf7.5*, which encodes a polypeptide of 63 amino acids. The inactivation of the gene led to a decrease in the cyanobacterial basal and acquired thermo-tolerances. Accumulation of the *groEL* transcript in the *orf7.5* mutant was strongly reduced. We postulate that the Orf7.5 protein together with the major sigma factor interacts with the *groEL* gene to regulate its heat induction.

1．熱ショック応答の発見

Ritossa は，ショウジョウバエの幼虫（あるいはその唾液腺組織）を通常の培養温度（25℃）から高温（30 ℃）に短時間（30 分間）さらすと，唾（液）腺多糸染色体の幾つかの特定の部位に膨らんだ構造（パフ，puff）が新たに誘導されることを観察し，1962 年に報告した（1）．このパフの生成は一過的で，温度が下がると消失した．一方，通常温度で存在したパフは，このような「熱ショック」により観察されなくなった．発生段階に特異的な変化をするパフは遺伝子の活性化（mRNA の合成）と関係があると当時既に考えられていたが，この論文はパフのパターンが温度などの外部環境の変化によって著しく，かつ再現性よく変化する（外部環境の変化が特定の遺伝子の活性化を引き起こす）と

いうことを示した点で重要である．温度が数度変化したときの唾液腺の巨大染色体を光学顕微鏡下で観察するという簡単な実験を行い，わずか3ページ（正味1ページ半）に纏められたこの論文は，現在データベース（PubMed）で熱ショックタンパク質（heat shock protein）をキーワードにして検索すると17,640件の論文がでてくるHSP研究の幕開になった．

2．熱ショックタンパク質の発見

Tissieresらは，当時開発されたばかりのSDS－ポリアクリルアミドゲル電気泳動法を用いて，ショウジョウバエの熱ショックに伴い合成されるタンパク質（放射標識されたアミノ酸を取り込んだタンパク質）を初めて分離・検出した（2）．6種類の主要タンパクは唾液腺以外の組織を熱ショック処理することによっても同様に誘導され，これらだけで放射標識された全タンパクの約30％を占めていた．熱ショックで合成が誘導されるこれらのタンパク質は，heat shock protein（HSP）と総称されることになった．さらにパフからHSPのmRNAが転写されることも確認され，熱ショック応答の研究は顕微鏡によるパフの観察に縛られることなく，転写産物やタンパク質を解析するという生化学的・分子生物学的研究へと発展していった．ショウジョウバエのみならず，大腸菌，酵母，哺乳類の細胞等でも熱ショック応答の研究が進み，熱ショック応答が種を超えた普遍的な現象であることが明らかにされた．1980年に塩基配列決定法が開発されると，HSP遺伝子が大腸菌，酵母，ショウジョウバエなどからクローニングされ，HSPタンパクのアミノ酸配列が比較された．その結果，HSPの一次構造が種を超えて驚異的な保存性を示すことが明らかになった．HSPは，アミノ酸配列の相同性に基づいて，HSP60あるいはシャペロニン60（大腸菌における相同タンパクはGroEL，以下同様に大腸菌などの原核生物における呼称を括弧内に示す），HSP70（DnaK），HSP90（HtpG），低分子量HSPなどのファミリーに分類される．HSPに続く数字を1000倍した値は，代表的メンバーのおおよその分子量である．HSPは，熱だけではなく，薬剤や他の物理化学的ストレスによっても同様に誘導されることから，ストレスタンパク質ともよばれる．

3．HSP遺伝子の発現制御

異なるHSP遺伝子は，熱ショックで一過的に発現する．HSP遺伝子発現のオン・オフはどのようにして起こるのだろうか．

高温感受性株などの変異株が既に分離され，分子遺伝学が進んでいた大腸菌を用いてHSP遺伝子の転写調節機構が詳しく解析された．熱ショックでHSPを発現できない変異株を用いたYuraやNeidhardtらの研究により，HSPの誘導は，HSP遺伝子の転写に特異的に必要とされるシグマ因子（σ^{32}）が熱ショックにより一時的に増加し，その結果このシグマ因子と結合したRNAポリメラーゼ（ホロ酵素）が増加することによって起こることが明らかにされた（3）．このシグマ因子は，異なるHSP遺伝子に共通に見いだされるプロモーター配列に特異的に結合する．このような「正」の調節に対して，通常温度におけるHSP遺伝子の転写がリプレッサーで抑制されるという負の機構が枯草菌などで最近明らかにされ，原核生物のHSP遺伝子発現調節機構の多様化が示唆された（4）．幾種かのシアノバクテリアのゲノムの全塩基配列が明らかにされているが，σ^{32}と相同な配列をコードする遺伝子（*rpoH*）はみつかっていない．我々がシアノバクテリアから初めてクローニングした低分子量HSP遺伝子の転写開始点上流には（通常の生育環境における遺伝子の転写を行っている）主要シグマ因子に認識される典型的なプロモーター配列が存在する（あるいは，σ^{32}のようなストレス特異的シグマ因子に認識される配列は見いだされない）にもかかわらず，通常温度では転写産物は検出されず，熱ショックにより mRNA の著しい蓄積が一過的に起こる（5）．このような結果から，我々は，非ストレス条件下で

はこの遺伝子の転写はリプレッサーにより抑制されていることを仮定した．実際，転写と翻訳開始点の中間には，未知のタンパク質が結合する，新規な逆位繰り返し配列（inverted repeat）が存在した（6）．この配列が，新規なリプレッサータンパク質が結合するオペレーターであるという作業仮説をたてて，この配列に結合するタンパク質の精製やその遺伝子のクローニングを行っている．

1995年にテキサスA&M大学のSusan Golden教授（Journal of Bacteriology のEditor）を訪問し，主にシアノバクテリアGroELに関する研究発表をしたが，教授からはHSP発現調節は既に大腸菌で明らかにされているのではないかと指摘された．しかし，今から振り返ればHSP研究を継続して良かったと思っている．負の調節の幕開となった，枯草菌のDnaKやGroEL遺伝子の転写抑制に関わるオペレーター配列CIRCE（7）を明らかにしたドイツのSchumann教授が1998年に我々の研究室を訪問された際に，教授も枯草菌のHSP研究を始めた頃同じような批判あるいは忠告を受けたと聞いた．さらに付け加えたい話は，今年のコールドスプリングハーバー研究所の分子シャペロンに関する会議で，大腸菌HSP遺伝子発現調節研究の第一人者である由良隆先生に初めてお会いし，大腸菌の熱ショック応答機構に関する質問をしたところ，先生はすかさず「よくわからないでしょう．実はよくわかっていないのです」と言われたことである．

4．分子シャペロン

HSPの細胞における機能は何か．HSPがタンパク質の折りたたみ（folding）に関与する「分子シャペロン（molecular chaperone）」であることが認められるようになったのは1980年代後半になってからであった．それまでに，Georgopoulosらによって大腸菌の代表的HSPであるGroELがファージ（細菌を宿主とするウィルス）の形態形成に関与することが明らかにされていた．大腸菌のGroELと，Ellisらが発見した植物のRubiscoサブユニット結合タンパク質（RsuBP）の遺伝子がクローニングされて，各々にコードされたタンパク質のアミノ酸配列が比較された（8）．その結果，約50％が同一のアミノ酸で，異なるアミノ酸でさえも類似のものであることが明らかになった．RsuBPは，Rubisco（リブロースービスリン酸カルボキシラーゼ，光合成の二酸化炭素の固定を行う酵素）が大（L）小（S）二つのサブユニットから成るLS複合体を形成するときに必要とされ，RsuBPは一時的にLサブユニットと複合体をつくるが，最終的には天然の機能的複合体（葉緑体ではL_8S_8）を形成したRubiscoから離れていく．GroELやRsuBPと相同なタンパク質は，大腸菌以外の原核生物やヒトを含む真核生物のミトコンドリアでもみつかりシャペロニン（chaperonin）と命名されることになった．生物種や細胞内局在性を超えた普遍性から，シャペロニンは，ファージの形態形成やRubiscoの高次構造形成に限られないもっと普遍的な（タンパク質の高次構造形成に関わる）機能を持っているのではないかと考えられた．紙面が限られているので詳細は省くが，現在では，変性タンパク質（あるいは構造上未熟なタンパク質）の天然構造（機能的立体構造）への折りたたみなどにシャペロニンが関与することが明らかになっている．他のファミリーのHSPにも同様の「シャペロン」としての働きがあることが明らかになっていった．1987年に，Ellisはタンパク質の正しい高次構造形成を助けるが，自らはその構造の最終成分にならないタンパク質を分子シャペロンと総称することを提案した．

細胞の中では，機能的な高次構造をもったタンパク質はポリペプチドの一つの存在形態にすぎない．未熟なタンパク質としては，リボソームにおける合成途中のポリペプチドがあげられる．ある程度の長さのペプチド鎖が合成されるまでは「球状」になれない．球状タンパク質は疎水的なアミノ酸の多い領域を内側に，親水的なアミノ酸の多い領域は分子表面に出るようにコンパクトに折りたたまれている．Foldingが不完全な場合には，疎水的相互作用によりポリペプチド同士は絡み合って凝集し固まってしまうかもしれない．たとえ合成されてしまったタンパク質でも，高温などのストレス条件下

で変性すると内部の疎水性領域が露出し，複数のポリペプチド間で疎水的な相互作用が働くことが考えられる．分子シャペロンは不可逆的な凝集や間違った folding を起こさないようにし適切な folding を可能にするものである．従って，ストレス下では細胞は多量の HSP を必要とする．一方，タンパク質の unfolding が生理的に重要な場合もある．サイトゾルで合成されたタンパク質はそれが機能する場（例えばミトコンドリアなどの細胞小器官）に正しく運ばれることが重要である．分子シャペロンは，膜を透過する前のタンパク質を透過しやすい，ほどけた状態（変性？状態）に保持し，さらに膜を透過してきたタンパク質の folding を助けることが明らかにされてきた．さらに，タンパク質の調節された分解や，（信じられないような話ではあるが）凝集してしまったタンパク質の可溶化にも分子シャペロンが関与することが明らかになっている．従って，分子シャペロンは，単なるシャペロン（若い女性の介添え後見役をする婦人）ではなく，医者として傷を癒したり一度絶命したものを蘇生させたり，さらには死体処理まで行うことが明らかになってきたわけである．

5．シアノバクテリアの HSP

シアノバクテリア（ラン藻あるいは藍色細菌ともよばれる）は，高等植物と同様に二種の光化学系からなる光合成電子伝達系をもち，水を分解して還元力を得る酸素発生型の光合成を行う原核生物である．この光合成の営みによって地球上に酸素が蓄積することになった．20 億年以上も前に地球に現れ，現在も地球上に広く分布しているシアノバクテリアは多様な環境（ストレス）に適応してきたと考えられる．我々は，HSP あるいはストレスタンパク質は，シアノバクテリアの環境適応や環境変化に対する馴化に，必須の役割を果たしてきたのではないかと考えて研究を進めている．以下に研究成果の一部を紹介する．大きな環境変化には曝されないと思われる大腸菌における HSP 研究に比べて，シアノバクテリの HSP 研究には未開拓の領域が多く残されている．

HtpG (HSP90)

HSP90 は真核生物の細胞内に大量に存在する（非ストレス時においても全可溶性タンパク質の約１％を占める）主要 HSP の一つであり，酵母やショウジョウバエでは必須のタンパク質であることが明らかにされている．変性タンパク質の凝集抑制に加え，平常時でも，（おそらく構造的に不安定な状態にある）多くの転写因子やシグナル伝達分子と複合体を形成し，これら標的タンパク質への結合・解離を通して広範な細胞機能を制御している．HSP90 は，細胞に十分に存在しているときには，いくつかの遺伝子に変異があっても表現型にならずにそれらの変異を潜在化させて蓄積しておくことができると考えられている（9, 10）．抗がん作用を持つ抗生物質ゲルダナマイシン（geldanamycin）が HSP90 を特異的に阻害することがわかり，抗がん剤の開発においても重要なタンパク質となっている．

グラム陰性の大腸菌やグラム陽性の枯草菌には HSP90 ホモログが HtpG しか存在しないが，欠損しても致死とはならないし，高温における生育や生存率に顕著な差が認められない．変異株の表現型が現れないために，HtpG の機能は不明であった．

我々は，HtpG 遺伝子をシアノバクテリア（*Synechococcus* sp. PCC 7942）から初めてクローニングした（11）．この遺伝子は，分子量 72,602，等電点 4.8 のポリペプチドをコードする．我々は大腸菌でこの遺伝子を大量発現させ，タンパクを精製し生化学的解析を行っている．ホモ二量体を形成する HtpG は，他のタンパク質の熱変性による凝集を阻止する活性を示した（図１）．HtpG に対する抗体を作製し，シアノバクテリア細胞抽出液中に HtpG を検出したが，真核生物の HSP90 のように細胞内に大量に蓄積しない（12）．HtpG 遺伝子破壊株を作製し，原核生物では初めて，HtpG が高温における増

殖や生存に必須のものであることを明らかにした．今年スイスで開催された第一回HSP90（The Hsp90 Chaperone Machine）国際会議でこれらの結果を発表したところ，HtpG（あるいはHSP90）が熱耐性獲得に関与することを初めて明らかにしたとの評価を受けた．細胞に致命的ではない穏やかな熱ショックを与えるとその細胞の熱耐性が著しく上昇することが知られている．これは，生物に普遍的に観察される現象であり，獲得性熱耐性（acquired thermotolerance）とよばれている．穏やかな熱ショックによるHSPの誘導が，その主要な原因であると考えられる．HtpG遺伝子破壊株では，この獲得性熱耐性が全くみられないので，HtpGは他のHSPに代替されない特別な役割を果たしていることが示唆された．HtpGタンパクは低温（16℃）や強光下でも蓄積し，HtpG遺伝子破壊株の生育や光合成活性が対照の野生株に比較してより強く阻害される（12，13）ことから，HtpG遺伝子は高温以外のストレス下でも重要な働きをしているに違いない．このシアノバクテリア変異株に，例えば部位特異的な変異を起こしたHtpG(あるいはHSP90)遺伝子を戻し野生型の表現型への回復の程度を解析することにより，このタンパクの構造と機能の関係を調べる予定である．

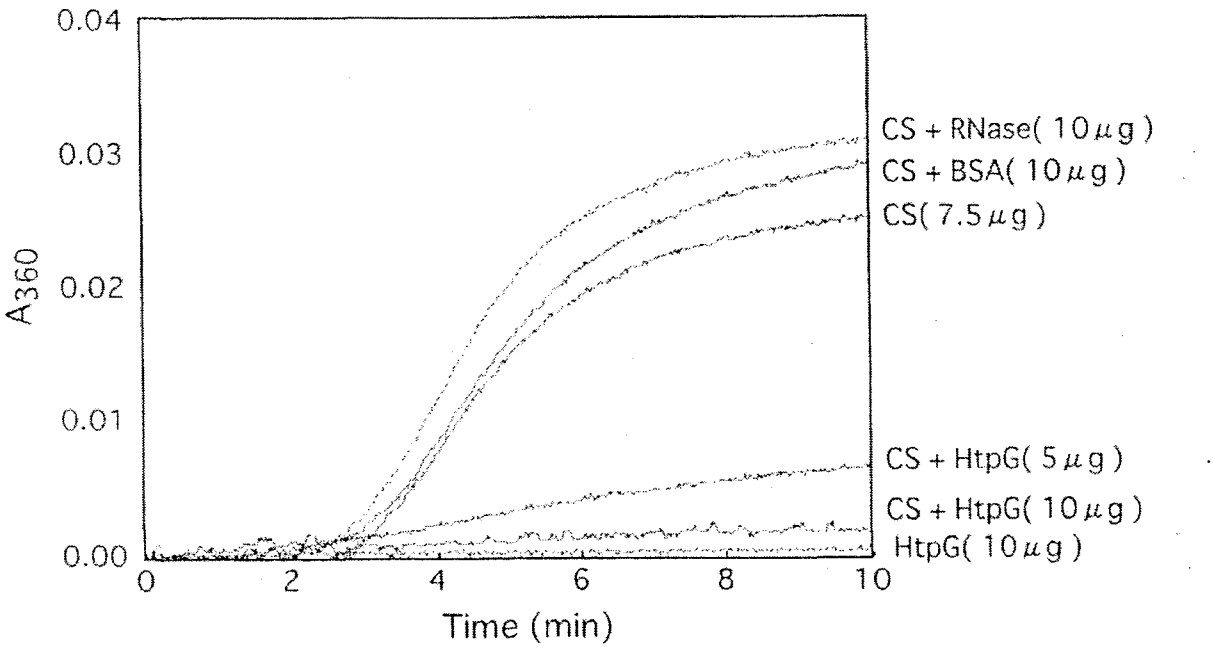

Fig.1. Suppression of thermal aggregation of citrate synthase (CS) by the purified recombinant HtpG. CS (7.5 μg) was mixed at 21°C with HtpG in 50 mM HEPES-KOH, pH 7.5 (total volume, 1ml), and then shifted to 50°C. Relative light scattering indicative of protein aggregation was measured as the apparent absorbance at 360 nm. For comparison, either ribonuclease A (RNase) or bovine serum albumin (BSA) was used instead of HtpG.

低分子量HSP（small HSP, small stress protein）

低分子量HSPは，その単量体の分子量が，12Kから42Kで，大きな多量体を形成する．HSP90，HSP70やHSP60などのHSPファミリーと比較すると，低分子量HSPのアミノ酸配列の保存性は高くない．ただし，カルボキシル末端側にα-クリスタリンドメインとよばれる比較的相同性の高い領域が存在する．哺乳動物ではHSP27，αA-及びαB-クリスタリンなど7個のホモログが確認されている．低分子量HSPは変性タンパク質と結合してその凝集を抑制し，折りたたみ可能な構造を保持して，HSP70などの他の分子シャペロン系に受け渡し，これらが変性タンパク質を天然構造に再生すると考えられて

いる．最も「なぞの多い」HSP であったが，最近になってようやく結晶構造や分子シャペロン機能の解析が進んできた．HSP27 は抗アポトーシスタンパク質であり，またアクチンの重合を阻止する．

我々がクローニングした好熱性シアノバクテリア（*Synechococcus vulcanus*）の small HSP 遺伝子（*hspA*）は，分子量 16,519，等電点 5.3 のタンパクをコードしていた（5）．シアノバクテリアにおいては，低分子量 HSP と GroEL は熱ショックで最も顕著に蓄積する HSP である（14）．この HSP はホモ 24 量体を形成し，他のタンパク質の熱による変性・凝集を抑制する活性を示した（14）．低分子量 HSP を構成的に大量発現するシアノバクテリア変異株を構築したところ，この変異株の致死温度における生存率（図 2），光合成電子伝達活性や光合成の集光機能を担うフィコシアニンの熱安定性が顕著に増加した（15）．これらの結果は高温ストレス下で，低分子量 HSP が，独立栄養生物に必須である光合成機能を防御することを示唆するものである．独立栄養生物や原核生物の低分子量 HSP と相互作用する標的タンパク質は不明であるが，我々は光合成関連タンパク質に絞り標的タンパク質を明らかにしようとしている．

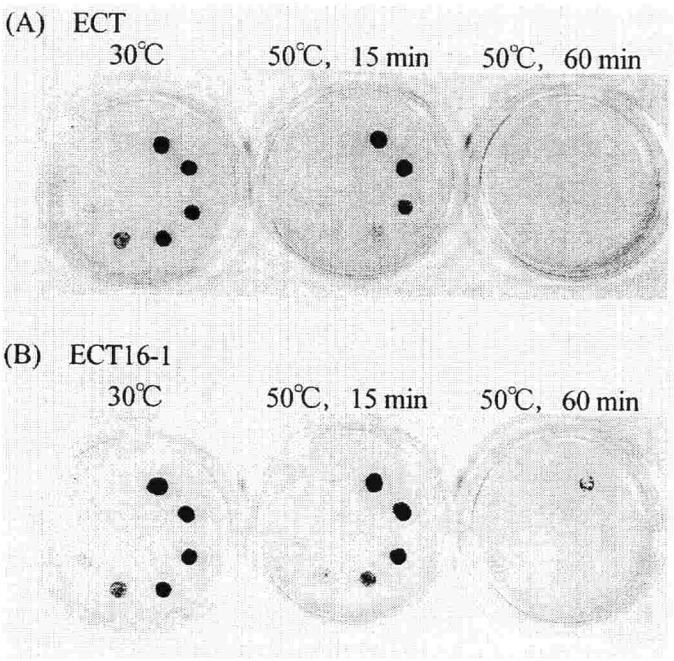

Fig.2. The basal thermo-tolerance of the reference strain (ECT, A) and the transformant strain (ECT16-1, B) which constitutively expresses the small HSP, HspA. Cultures of the ECT and ECT16-1 strains grown at 30°C were shifted directly to a 50°C-water bath for either 15 or 60 min in the light. An aliquot of the culture before or after the heat treatment was serially diluted (1:1, 1:5, 1:25, 1:125, 1:625, 1:3125, 1:15,625, 1:78,125) and a 10-μl aliquot from each dilution was spotted onto each section on a BG-11 agar plate. The plate was incubated at 30°C for two weeks.

新規な HSP

シアノバクテリア（*Synechococcus* sp. PCC 7942）から，熱ショックで誘導される新規な HSP 遺伝子をクローニングし，これにコードされるタンパク質を Orf7.5 と命名した（16）．Orf7.5 は，分子量 7,455，等電点 5.0 のポリペプチドで，筆者の知る限りにおいて世界最小の HSP である．Orf7.5 の一次構造と相同な配列はデータベースに見出されない．Orf7.5 遺伝子破壊株を作製し，この遺伝子が高温における増殖・生存に重要であることを明らかにした．この変異株では，GroEL 遺伝子の発現が著

しく阻害されていた．東京農業大学の吉川博文教授と荷村かおり博士は酵母２ハイブリッド法を用いて遺伝子ライブラリをスクリーニングし，Orf7.5と主要シグマ因子が特異的に相互作用することを発見した（未発表）．Orf7.5により通常の転写をつかさどる主要シグマ因子がストレス特異的シグマ因子に転換するのではないかという新しい作業仮説をたてて，吉川教授や東京大学の田中寛博士と共同で研究を進めている．

謝辞：ここに記したすべての研究成果は，筆者が埼玉大学で卒業論文・修士論文及び博士論文の研究指導を行った学生諸君と共同で行ったものであり，この場を借りて彼らに深く感謝申し上げる次第である．筆者が最も困難な状況にあった研究開始当時，年末年始の休みさえも惜しんで忍耐強くHSPや光合成関連の研究に没頭する彼らに何度も励まされた．

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論文投稿中

Title

Light plays a key role for the modulation of heat shock response in cyanobacteria

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Summary

The heat shock response is generally characterized by a rapid, intense and transient activation of gene expression, resulting in the elevated synthesis of a family of proteins. We found that light modulates these characteristics of the heat shock response in cyanobacteria. Light accelerated the heat induction of *htpG, groESL1, groEL2,* and *hsp17*, in *Synechocystis* sp. PCC 6803 as well as that of *groESL* and *htpG* in *Synechococcus* sp. PCC 7942. In both strains, the maximum accumulation of transcripts of these genes was observed when cells were subjected to continuous illumination prior to and during heat shock, although each heat shock gene responded rather uniquely. Except for *hsp17*, no mRNAs of the heat shock genes were detected in dark-treated cells prior to and during heat shock. The time-course for the appearance of mRNA after heat shock in the light varied, depending on the heat shock gene. In darkness, the heat shock response of all the heat shock genes tested was slower than in the light and it was not transient. There was an apparent relationship between the enhancement of the heat-shock gene transcription in the light and the level of reduced plastoquinone in the photosynthetic electron transport system. Light did not affect the mRNA stability of the heat shock genes in *Synechocystis* sp. PCC 6803 at least during the early phase of the heat shock response, although the stability was quite different, depending on the heat shock gene. Light also enhanced the accumulation of the GroEL protein and the acquired thermo-tolerance.

Introduction

The heat shock response represents a ubiquitous protective and homeostatic cellular response to cope with heat-induced damage to proteins (Yura et al., 2000). Many heat shock proteins (HSPs) are molecular chaperones or proteases and play major roles in protein folding, assembly, refolding, degradation, and translocation under normal and stress conditions.

The heat shock response is generally characterized by a rapid, intense and transient activation of gene expression, resulting in the elevated synthesis of a family of HSPs. Heat shock response has been extensively studied in *Escherichia coli* in which induction of HSPs occurs primarily by transient increase in the sigma 32 factor (Yura et al., 2000). This leads to the transcription of more than 30 genes from the conserved heat shock promoters by combining the RNA holoenzyme with sigma 32, thus constituting a heat-shock regulon. In addition to positive control by the use of alternative sigma factors such as sigma 32, negative control of HSP gene expression by specific repressors operates in eubacteria other than *E. coli* (Narberhaus, 1999). The most wide spread and best studied negative regulation is the CIRCE/HrcA system. CIRCE, controlling inverted repeat of chaperone expression (Zuber and Schumann, 1994), is an inverted repeat (IR) consisting of 9-bp separated by a 9-bp spacer, which has been found in more than 40 different bacterial species and shown to act as a negative *cis*-element of *dnaK* and/or *groESL* operons (Narberhaus, 1999). The HrcA repressor protein interacts with CIRCE (Yuan and Wong, 1995; Schulz and Schumann, 1996).

Cyanobacteria are autotrophic Gram-negative prokaryotes having a plant-type oxygen evolving photosynthetic apparatus. Upon a temperature upshift, cyanobacteria induce a set of HSPs (Borbély et al., 1985; Webb et al., 1990) among which GroEL, small HSP and GroES are the most prominent HSPs which accumulate in the cell (Roy et al., 1999). Mutational analysis has shown that ClpB, HtpG, and small HSP play an important role in high temperature tolerance (Eriksson and Clarke, 1996; Lee et al., 1998; Tanaka and Nakamoto, 1999; Nakamoto et al., 2000).

Although sequences similar to the heat shock promoters of *E. coli* were found upstream of the *groESL* operon in *Synechococcus* sp. PCC 7942 (Webb et al., 1990), there is no evidence for an alternative sigma factor controlling the heat shock regulon in cyanobacteria. CIRCE has been identified around the transcriptional start sites of the two *groEL* genes in *Synechocystis* sp. PCC 6803 (Glatz et al., 1997). In cyanobacteria, only *groEL* genes, but not *dnaK* genes, have the CIRCE. Two distinct *groEL* genes generally exist in a cyanobacterial cell (Chitnis and Nelson, 1991; Lehel et al., 1993, Furuki et al., 1996; Tanaka et al., 1997). One gene designated as *groEL1* is co-transcribed with *groES*, while the other, *groEL2* or *cpn60*, does not form an operon with *groES* and produces a monocistronic mRNA. In the genome of *Synechocystis* sp. PCC 6803, an open reading frame which encodes a homologue of HrcA is present (Kaneko et al., 1996). Thus, the CIRCE/HrcA system may regulate the *groEL* genes in the cyanobacterium, although it is yet to be proven. The CIRCE/HrcA system is not the only mechanism which controls HSP genes in cyanobacteria. Recently, we identified a novel DNA element in a 5'-untranslated region of the *hspA* gene in the thermophilic cyanobacterium *Synechococcus vulcanus* with which a DNA-binding protein specifically interacts (Kojima and Nakamoto, 2002). We proposed that the DNA element is a specific target for a DNA-binding protein and that it plays a role in the regulation of expression of the cyanobacterial *hspA* gene which encodes a small HSP homologue.

Heat shock enhanced accumulation of transcripts of the two *groEL* genes, *groESL1* and *groEL2*, in *Synechocystis* sp. PCC 6803 in the light, but induction was lower in the dark (Glatz et al., 1997). The HSP induction by light indicates that light combined with high temperature may induce stressful conditions in cyanobacteria. Light energy is used to drive the electron transfer from water to $NADP^+$ and to produce ATP. It is essential for the life of photoautotrophs, but excess light energy is harmful when it generates reactive oxygen species by the electron transport systems. Light energy may become excess even under low irradiance as well as high irradiance since a balance between energy supply by light harvesting and electron transport and consumption by CO_2 fixation and nitrogen assimilation will determine the extent of 'excess'. We showed that the survival rate of *Synechococcus* sp. PCC 7942 was much less when heat shock was given under (low) irradiance than when given in darkness (Nakamoto et al., 2000). Thus, photoautotrophs such as cyanobacteria and higher plants might have developed a unique control mechanism to induce heat shock genes by using light as well as temperature as a signal in order to avoid damage induced by excessive heat and light excitation.

The present study was conducted to examine the effect of light on the heat-induced transcriptional activation of heat shock genes in *Synechocystis* PCC 6803 and *Synechococcus* PCC 7942. We have also studied the effect of light on the mRNA stability of heat shock genes and on the HSP accumulation. Further evidence for the physiological importance of the light activation of the heat shock gene expression was obtained by enhanced acquired thermo-tolerance in the light.

RESULTS

Effect of light on the transcription of different HSP mRNAs from Synechocystis sp. PCC 6803 and Synechococcus sp. PCC 7942

In order to study whether the amplification of the cyanobacterial heat-shock response by light is a general phenomenon, we determined whether the heat-shock induction of a gene other than *groEL*s is also stimulated by light. *Synechocystis* sp. PCC 6803 cells were grown at 30°C and under the light intensity of 35 $\mu E/m^2/s$. Cells were pre-incubated at 30°C for three hours in the light or dark, then heat shocked at 42°C or left at 30°C either with or without illumination for 15 min. After each treatment, the expression of *htpG* and *hsp17* as well as the two *groEL*s was analyzed by Northern blot analysis using a DIG-labeled gene-specific RNA probe. The light intensity utilized in the present study was 35 $\mu E/m^2/s$, the same as used in growth of cells. All the probes except for the *hsp17*-specific probe hybridized with multiple RNA species. The *htpG*-specific probe hybridized with 2.3- and 1.4-kb mRNAs. The *groEL2*-specific probe hybridized with 1.9- and 1.4-kb mRNAs, while the *groESL1*-specific probe hybridized with 2.3-kb mRNA as a major one.

Light, prior to and/or during heat shock, exerted a significant effect on the heat induction of the *htpG*, *groESL1*, *hsp17*, and *groEL2* genes from *Synechocystis* sp. PCC 6803 (Fig. 1A). These effects were specific, since a housekeeping gene, such as *rpnB*, did not respond to light conditions or to temperature changes (data not shown). When cyanobacteria were pre-dark-treated at 30°C and subsequently heat-shocked at 42°C in darkness, the *htpG*, *groESL1*, and *groEL2* genes were not heat-induced (Fig.1A, 30D-42D). On the other hand, the greatest accumulation of mRNA was detected when both the pre-heat-shock and heat-shock treatments were performed in the light (Fig. 1A, 30L-42L). The irradiation prior to the heat-shock treatment enhanced significantly the heat induction of all four heat shock genes when cells were heat-shocked in the dark (Fig. 1A, compare '30D-42D' with '30L-42D'). Only the *hsp17* gene was induced upon heat shock in the dark after the pre-heat-shock treatment in the dark (Fig.1A, 30D-42D). The presence of light during the pre-heat-shock and/or heat-shock treatments further increased the mRNA accumulation of the *hsp17* gene (Fig.1A). The results indicated that the extent of the light effect on the heat induction varies depending on the heat shock gene. Light elevated the steady state level of the *groEL2* mRNA under normal conditions at 30°C (Fig.1A, 30L).

In order to examine whether the enhancement of the heat induction of the HSP genes by light is commonly observed among different cyanobacterial species, we performed similar experiments with *Synechococcus* sp. PCC 7942 which is a unicellular cyanobacterium like *Synechocystis* sp. PCC 6803. In this cyanobacterium, only one *groEL* gene in *groESL* operon has been reported (Webb et al., 1990). No gene encoding a small HSP homologue has been cloned yet. Thus, we analyzed the expression of only the *htpG* gene and *groESL* operon. In *Synechococcus* sp. PCC 7942, light exerted a similar effect on the expression of the *htpG* and *groESL* genes (Fig. 1B). The accumulation of both the *htpG* and *groESL* mRNAs was greatly enhanced only when the heat shock was performed in the light (Fig. 1B, 30D-45L and 30L-45L). The heat shock response under various conditions by *Synechococcus* sp. PCC 7942 was not the same as that by *Synechocystis* sp. PCC 6803. In *Synechocystis* sp. PCC 6803, the light conditions both prior to and during heat shock affected the level of the *htpG* and *groESL1* mRNA accumulation. On

the other hand, the light conditions only during heat shock are effective in enhancing the heat induction in *Synechococcus* sp. PCC 7942. Thus, there is some variation in the effect of light between species.

In the following experiments, we used *Synechocystis* sp. PCC 6803 cells which were cultured at 30°C in the light.

Effect of light on the time course of the mRNA accumulation of the heat shock genes

Since the light conditions for a fixed period of heat shock showed a significant effect on the accumulation of mRNAs of all four heat shock genes, we decided to follow the expression of the heat shock genes in light or darkness during a 3-h period after heat shock.

Fig. 2 shows the kinetics of high temperature-induced accumulation of the HSP mRNAs either in light or darkness. In the light, all the heat shock genes showed a transient accumulation of mRNA during heat shock, although the kinetics of induction was different for these HSP mRNAs. The mRNA accumulation of the *htpG* and *hsp17* genes reached a maximum level 10 to 30 min after a temperature shift-up, while those of the two *groEL* genes reached a maximum after 30 to 60 min. The *htpG* gene appears to reach a maximum earlier than the *hsp17* gene. Thus, the time-course for each heat shock gene or group of heat shock genes varies. These results suggest the possible involvement of multiple regulatory mechanisms for the heat induction of these heat shock genes.

In the dark, the heat induction of all the genes was slower than that in the light and significant lags were detected in the *hsp17* and *groESL1* gene induction. Except for *htpG*, there was no transient peak in the level of mRNA accumulation within three hours. In the dark, the level of the *hsp17* mRNA reached the maximum 60 min after heat shock, and maintained a high level for an additional two hours. The two *groEL* genes kept accumulating mRNAs. Thus, light may be required for long-term feedback inhibition of the heat shock gene expression in cyanobacteria.

Effect of photosynthetic electron transport inhibitors on the transcription of the heat shock genes

To test whether the activation of the transcription of the *hsp17*, *groEL*, and *htpG* genes are linked to the operation of the photosynthetic electron transport, we examined the effect of DCMU on the heat-induced accumulation of mRNA in the light (Fig. 3). Glatz et al. (1997) showed that DCMU inhibits the mRNA accumulation of the two *groEL* genes. We confirmed their results and further showed that the light-induced accumulation of the *htpG* mRNA is strongly inhibited by diuron. Similar results were obtained with *htpG* and *groESL* genes from *Synechococcus* sp. PCC 7942 (data not shown). However, *hsp17* was transcribed normally in the presence of 15 μM DCMU, a concentration high enough for the complete inhibition of the photosynthetic oxygen evolution (data not shown). In order to localize the site of the electron transport chain which is involved in the transcription of the heat shock genes directly or indirectly, we examined the effect of DBMIB on the transcription in the light. DCMU inhibits electron flow from photosystem II into the plastoquinone pool, while DBMIB inhibits oxidation of plastoquinone by photosystem I. 10 μM DBMIB which inhibited the photosynthetic oxygen evolution by more than

95% did not inhibit the light-induced accumulation of the *htpG* and *groEL2* mRNAs. Similar results were obtained in *groESL1* (data not shown). DBMIB appeared to stimulate the accumulation of the *htpG* mRNA. These data are consistent with the involvement of redox state of plastoquinone pool in the heat induction of cyanobacterial *groEL* and *htpG* genes. When plastoquinone is in a reduced state, the transcription of the *htpG* and *groEL2* genes is enhanced. In contrast to these genes, the *hsp17* mRNA level was not affected by the inhibitors.

Effect of light on the stability of different HSP mRNAs

Synechocystis sp. PCC 6803 cells which had been pre-treated at 30°C in the light was heat-shocked at 42°C for 15 min, and then either kept in the light or transferred to dark for 5, 10, or 20 min at 42°C. The level of the *htpG* transcripts was reduced to a negligible level within 5 min after the dark shift as compared to the corresponding light-treated sample for the same time interval (Fig. 4, A). The mRNA levels of the *groESL* operon and the *groEL2* gene showed significant reductions, but the accumulation of the *hsp17* mRNA was reduced only slightly (Fig. 4, A). The reduction of the mRNA accumulation after the dark shift may be due to the reduction of the transcriptional activity and/or that of the mRNA stability.

In order to examine the effect of light on the stability of mRNA, the transcription initiation inhibitor rifampicin was added after heat shock at 42°C for 15 min, and then cultures were incubated either in the light or in the dark for 5, 10, and 20 min (Fig. 4, B). The *htpG* mRNA was found to be the most unstable mRNA among the heat shock genes tested. The *htpG* transcripts were undetected within 5 min after the rifampicin addition both in the light and dark. In contrast to the *htpG* mRNA, *hsp17* mRNA transcripts did not show any significant change in the mRNA level during these treatments. The *groEL2* mRNA was less stable than the *groESL1* mRNA. Only the *groESL1* transcript showed some difference in its stability due to the light conditions. It appeared to be slightly more stable in the light than in the dark. The results indicate that light may not play a role in determining the HSP mRNA stability during the early phase of the heat shock response, but it exerts its effect on the transcription of the heat shock genes.

In order to examine whether the photosynthetic electron transport system influences the level of HSP mRNAs, we added DCMU to a culture after heat shock and then the culture was incubated in the light (Fig. 4, A). As a control, the same volume of ethanol which was used as a solvent for the DCMU stock solution was added instead of DCMU. The DCMU addition appeared to have a similar effect to the dark shift, supporting the idea that the photosynthetic electron transport system regulates the transcription of the HSP genes. However, compared with the dark shift, the effect of DCMU on the level of the *groEL2* mRNA was smaller.

The effect of DCMU on the stability of the HSP mRNAs was examined by determining the change in the level of mRNA after the addition of rifampicin in the presence of DCMU or ethanol (Fig. 4, B). We did not detect any significant change in the stability of all the mRNAs by the presence of DCMU. The results indicate that the photosynthetic electron transport system may not affect the stability of the HSP mRNAs and that the rapid and intense heat response of heat shock genes in cyanobacteria is achieved by the transcriptional activation in the light. The light signal for the transcriptional activation of heat shock genes except *hsp17* is mediated by the photosynthetic electron transport.

A unique feature of the heat-shock response in the dark is the absence of a transient peak in the mRNA levels of the *hsp17* and two *groEL* genes. In the light, these genes decrease their mRNA levels 60 min after heat shock, while there was no reduction in these mRNAs during a 3-h period after heat shock in the dark (Fig. 2). This may be due to higher rate of transcription and/or lower rate of mRNA degradation in the dark than in the light. In order to examine the activity of mRNA degradation, we added rifampicin 60 min after heat shock and followed the decrease in the mRNA level up to three hours after heat shock (Fig. 5). At the same time, the mRNA level in cells of an aliquot of the same culture containing no rifampicin was followed in order to deduce the transcriptional activity. If we assume that rifampicin does not change the rate of the mRNA degradation, the net difference between the mRNA levels in the absence and presence of rifampicin is due to the de novo synthesis of mRNA. In the light, the transcription of *hsp17* appears to become very low two hours after heat shock since there is no significant difference between the cellular mRNA levels in the presence and absence of rifampicin at that time point (Fig. 5). However, in the dark, the transcriptional activity was kept high up to three hours after heat shock (Fig. 5). The mRNA stability appeared to be greater in the dark than in the light. Thus, the lack of reduction of the *hsp17* mRNA level up to three hours in the dark (Fig. 2) is due to the continued synthesis of the *hsp17* mRNA and greater stability of mRNA. In contrast to *hsp17*, the transcription of the *groESL1* and *groEL2* genes was very active up to three hours after heat shock both in the light and darkness (Figs. 5). Although the transcription of the two genes was active, the rate of the transcription started to decrease earlier in the light than in the dark. Similar to *hsp17*, these mRNAs were more stable in the dark than in the light, when the RNA synthesis was stopped 60 min after heat shock. We suggest that no decrease or some increase in the level of the *groESL1* and *groEL2* mRNAs (Fig. 2) is achieved by the prolonged transcription and the stabilization of mRNAs. The response of the *htpG* mRNA appears to be similar to the *groEL2* mRNA.

Effect of light on the accumulation of HSP upon a heat shock

The effect of light on the heat induction of HSPs was investigated by SDS-PAGE and Western blot analysis. Cells grown at 30°C were treated in the presence or absence of light at 42°C for one hour, and then transferred to 48°C for one hour either in the presence or absence of light. Soluble proteins were extracted from cells at each step of the heat-treatment. The accumulation of a 60-kDa protein was greatly enhanced when cells were heat-shocked at 42°C in the presence of light (Fig. 6A, 42L). The protein accumulated further during the subsequent lethal temperature (48°C) treatment in the light, but not in the dark (Fig. 6A, compare '42L-48L' with '42L-48D'). No accumulation of the protein was detected when cells were directly shifted to the lethal temperature (Fig. 6A, 48L). This enhanced accumulation of the protein was not observed when the heat-shock treatment at 42°C was performed in the dark, irrespective of the additional lethal temperature treatment in the light or dark (Fig. 6A). The 60-kDa protein must be GroEL based on the heat induction and the size of the protein. Western blot analysis with polyclonal antibodies against GroEL confirmed that the accumulation of GroEL increased greatly by irradiation during the heat shock at 42°C (Fig. 6B, 42L) and was further enhanced by the subsequent lethal temperature treatment in the light (Fig. 6B, 42L-48L). No enhancement was detected when cells were directly shifted to 48°C in the light (Fig. 6B, 48L), or pre-incubated at 42°C in the dark and then shifted to 48°C in the light or dark (Fig. 6B, 42D, 42D-48L, and 42D-48D). Western blot analysis with polyclonal

antibodies against DnaK showed that its accumulation was slightly enhanced when cells were pre-incubated at 42°C irrespective of the presence or absence of light (Fig.6B).

Effect of light on the acclimation to lethal temperature

Light during heat shock at 42°C was found to be a crucial parameter for the induction of mRNAs of at least several heat shock genes. Light also exerted a significant effect on the enhancement of the GroEL accumulation during heat shock. Thus, we expected that light may play an important role in the acquired thermo-tolerance.

Cells were treated in the same way as described above for the protein analysis. When cells were directly shifted to 48°C in the light, the survival rate was decreased to less than 0.01% (Fig. 7B, 48L). However, when cells were pre-treated in the light at 42°C, the survival rate after the lethal temperature treatment in the light or dark increased to 100 to 10,000 folds as compared with the direct shift (Fig. 7B, 42L-48L and 42L-48D). This acquired thermo-tolerance was significantly lower when the pre-treatment was performed in the dark. When cells were pre-treated in the dark, the survival rate after the lethal temperature treatment in the light or dark increased to 10 to 100 folds as compared with the direct shift (Fig. 7B, 42D-48L and 42D-48D). As we showed previously with *Synechococcus* sp. PCC 7942 (Nakamoto et al., 2000), *Synechocystis* sp. PCC 6803 cells acquired more thermo-tolerance when a lethal temperature treatment was performed in the absence of light than in the presence of light [Fig. 7B, compare '42L-48L ('42D-48L') with '42L-48D' ('42D-48D')].

DISCUSSION

The ability of photoautotrophic organisms such as cyanobacteria to detect and adapt to qualitative and quantitative changes in light is essential for their survival. Ultraviolet light and/or high irradiance of visible light are examples which threaten their lives. HSPs such as ClpP and HtpG in cyanobacteria have been shown to play an important protective role in the acclimation to these stresses (Porankiewicz et al., 1998; Hossain and Nakamoto, 2003). Recently, we have shown that even weak light is harmful under heat stress (Nakamoto et al., 2000). Thus, we expected that cyanobacterial heat shock response is modulated by light.

We have studied the effect of light under heat stress on the expression of HSPs, and found that light plays a key role for the maximal elevation in the transcript level of all *Synechocystis* and *Synechococcus* HSP genes examined during this study (Fig. 1). However, light prior to and/or during heat shock appears to have a differential pattern of control on each HSP gene. The control is delicate enough to lead to a differential expression of the two *groEL* genes (Fig. 1, see also Glatz et al., 1997). The differential expression is rather unexpected since the two *groEL* genes are thought to be controlled transcriptionally by the same CIRCE/HrcA system. An additional transcriptional control or a posttranscriptional control may be involved in the expression of the genes. The difference in the mRNA stability between *groESL1* and *groEL2* (Figs. 4 and 5) may be one of the reasons for the differential expression.

We found that the response by *hsp17* is unique in the light-independent heat induction. There was a substantial mRNA accumulation in cells pre- and heat-treated in the dark (Fig. 1A). The operation of the photosynthetic electron transport did not have a significant effect on its expression (Fig. 3). Thus, the regulation of the *hsp17* gene expression is distinguished from that of the gene expression of other HSPs examined in the present study. It is suggested that HSP17 stabilizes heat-stressed membranes (Török et al., 2001). HSP17 may play a unique role regardless of the presence or absence of light to protect thylakoid and/or plasma membranes under heat stress.

Light exerted a remarkable effect on the time course of the HSP mRNA accumulation. The transient accumulation could be observed only when cells were heat-shocked in the light (Fig. 2). In the dark, the two *groEL* genes kept accumulating mRNAs even three hours after heat shock. The continuous accumulation of the mRNAs was due to its continued synthesis and stabilization in the dark as shown in Fig. 5. The HrcA repressor negatively regulates expression of the *dnaK* and the *groEL* operons by binding to an operator CIRCE. It is shown in *Bacillus subtilis* that the activity of HrcA is modulated by the GroE chaperonin machine (Mogk et al., 1997). GroEL is necessary to activate HrcA. A thermal upshift generates an increase in the concentration of non-native proteins, which transiently deplete the free pool of GroEL proteins. Since GroEL proteins are necessary for activating HrcA, depletion of cells for GroEL will lead to an increase in inactive HrcA and thereby result in induction of the *dnaK* and *groEL* operons. They considered GroEL as the cellular thermometer in *Bacillus subtilis*. In cyanobacteria, upon heat shock in the light, HrcA is inactivated as described above for *Bacillus subtilis*. Then, the synthesis of GroEL proteins as well as other HSPs is enhanced and cells may establish a new steady state at the high temperature. Then the free pool of GroEL increases to activate HrcA, shutting off the *groEL* transcription. However, as shown in Fig. 6, the accumulation of GroEL proteins is inhibited in the dark, leading to prolonged depletion of GroEL proteins. Thus, the feedback inhibition of the *groEL* transcription may not take place efficiently in the dark. Although nothing is known about the regulation of the *hsp17* gene, we assume that an insufficient accumulation of HSP proteins in the dark may prevent a cell from having a feedback inhibition, leading to no transient gene expression in the dark. The GroEL protein may also feedback-inhibit the *htpG* transcription. In an *hrcA* disruptant, which constitutively express the GroEL proteins, the accumulation of the *htpG* transcripts was inhibited (H. Nakamoto, M. Suzuki, K. Kojima, unpublished).

Our present studies with the inhibitors DCMU and DBMIB of the photosynthetic electron transport showed the involvement of redox state of plastoquinone pool in the heat induction of cyanobacterial *groEL* and *htpG* genes. When plastoquinone is in a reduced state, the transcription of the two genes is enhanced. The inhibition pattern is very similar to that in the RNA helicase expression in *Synechocystis* sp. PCC 6803 (Kujat and Owttrim, 2000). Since all the HSP genes tested in this study were transcribed during heat shock even in the presence of DCMU which is enough to inhibit the photosynthetic electron transport system completely, the redox state of plastoquinone pool may not act as an on/off switch. Under physiological conditions, the level of the plastoquinone reduction is influenced by the level of consumption of light energy absorbed by pigments. We propose the following working models for the light modulation of heat shock response in cyanobacteria. Under high temperatures, the activity of anabolism such as photosynthetic CO_2 fixation and nitrogen assimilation would be reduced. This may lead to a decrease in the ratio of the photon energy used for the physiological processes and thus a situation where cells are exposed to photons in excess of its utilization capacity. The situation favors reduction of plastoquinone pool. The signal may be transduced to an unknown transcription factor which

modulates transcription of the heat shock genes. The situation where cells are exposed to photons in excess of its utilization capacity also favors generation of reactive oxygen. Thus, the oxidative stress caused by high temperature may become a signal which is transduced to enhance the expression of the heat shock genes.

Light or the operation of the photosynthetic electron transport system does not appear to exert a significant effect on the stability of HSP mRNAs in heat-shocked cells when the stability was examined 15 min after heat shock (Fig. 4). However, the *hsp17* and *groEL* mRNAs became more stable 60 min after heat shock in dark-treated cells than in illuminated cells (Fig. 5). In both cases, each HSP mRNA showed a greatly variable stability.

Light exerted its effect on the accumulation of the GroEL protein (Fig. 6). This corresponds to the enhanced accumulation of the *groEL* mRNA in the light. Heat shock may enhance the accumulation of HSPs other than GroEL. Light is the energy source to produce ATP. Thus, light may stimulate the translation of heat shock genes such as *groEL* by the increase in ATP level, although it may exert its effect on translation by other means. We have verified the effect of light on the transcription/translation of heat shock genes by analyzing the effect of light on the acquired thermo-tolerance. Cells acquired more thermo-tolerance when they were pre-treated at 42°C for one hour in the presence of light than in the absence of light (Fig. 7). The cells acquired thermotolerance in the translation machinery as well as in their viability at a lethal temperature, leading to a further accumulation of GroEL at the lethal temperature in the light. In summary, light plays a vital role in conferring a heat shock response in cyanobacteria which is rapid, intense and transient.

EXPERIMENTAL PROCEDURES

Organisms and culture conditions

Two cyanobacterial strains *Synechocystis* sp. PCC6803 and *Synechococcus* sp. PCC7942 were grown photoautotrophically in the BG-11 inorganic liquid medium or on BG-11 plates containing 1.5% (w/v) agar and 0.3% (w/v) sodium thiosulfate (Rippka et al., 1979). The BG-11 culture medium was modified to contain 20 mM N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) adjusted to pH 8.0 with KOH and 50 mg/L Na_2CO_3. The liquid cultures in glass vessels were incubated at a 30°C-water bath, continuously aerated, and illuminated with a light intensity of 35 $\mu E/m^2/s$. The electron transport chain inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) was dissolved in ethanol at 15 mM, while 2,5-dibromo-3-methyl-6-isopropyl-benzoquinone (DBMIB) was dissolved in 10% dimethyl sulfoxide in ethanol at 10 mM. These stock solutions were added to a culture in a 1/1000 dilution. The 30 mg/ml rifampicin stock solution was dissolved in methanol.

Heat-shock conditions

A photoautotrophically growing culture as described above was transferred to a pre-warmed glass vessel in a 42°C- or 45°C-water bath, and continuously aerated under the same light intensity. For a dark treatment, the glass vessel as well as the connecting tubes for aeration was covered with aluminum foil.

Preparation of total RNA

Cells that had been exposed to various experimental conditions were immediately collected by centrifugation and frozen in liquid N_2. Alternatively, cells were killed instantaneously by the addition of equal volume of 10% phenol in ethanol to the cell suspension, collected by centrifugation, and frozen in liquid N_2 (Kanesaki et al., 2002). Total RNA was extracted as previously described (Nakamoto and Hasegawa, 1999).

Northern blot analysis

Suitable fragments for the preparation of the *htpG*-, *groEL2*-, *groES*- (to detect transcripts of the *groESL* bicistronic operon), and *hsp17*-specific probes for detection of corresponding mRNA from *Synechocystis* PCC 6803 were amplified by polymerase chain reaction (PCR) with a template of the genomic DNA and appropriate pairs of primers listed in Table 1. In the case of *htpG*, *groEL2*, and *groES*, PCR fragments were cloned in either the pT7Blue T-vector (Novagen) or pBluescript II SK (Stratagene), linealized by the digestion with an appropriate restriction enzyme, and then digoxygenin-labeled complementary RNA was synthesized in vitro by T7 RNA polymerase. For the amplification of a DNA fragment containing portion of the *hsp17* gene, the antisense oligonucleotide primer containing the T7 RNA polymerase promoter was used (Table 1). Thus, it was directly used for the synthesis of the digoxygenin-labeled RNA probe. The *htpG*- and *groEL*-specific probes for detection of corresponding mRNA from *Synechococcus* sp. PCC 7942 were prepared as described previously (Nakamoto et al., 2001). 1μg of the RNA from the same preparation was loaded for each lane of a 1% agarose gel. After electrophoresis, RNA was blotted onto the BM positively charged nylon membrane (Boehringer Mannheim GmbH), and cross-linked. A separate blot was simultaneously hybridized with a different probe as instructed by the manufacturer (Boehringer Mannheim GmbH). After washing the membrane, chemi-luminescence signal from each probe was detected by exposing each blot together for different time intervals to X-ray films.

Protein extraction

Cells from culture were harvested by centrifugation at room temperature for 5 min at 3,000 rpm in Beckman GS-6 table-top centrifuge. The cell pellet was collected, and used immediately. The pellet was washed with 500 μl of 10 mM Tris-HCl, pH 8.0, and 1 mM EDTA. The cells were resuspended in 25 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, and 1 mM each of phenylmethylsulfonyl fluoride, benzamidine and caproic acid, and 3M urea. All of the following procedures were carried out at 4°C. The cell suspension was mixed with equal volume of glass beads (Sigma) to the volume of the cell pellet and disrupted by vortexing vigorously for 3 min. This process was repeated three times with a 3-min interval on ice between the vortexing, and the resulting suspension was centrifuged at 16,000 g for 30 min. The supernatant fraction was used for SDS-PAGE, and Western blot analysis.

SDS-PAGE

Equal amount of crude soluble protein was boiled for 5 min in a mixture containing 50 mM Tris-HCl, pH 6.8, 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, and 10% glycerol and then loaded onto a 15% polyacrylamide gel in the presence of SDS (Roy et al., 1999).

Western blot analysis

Western blot analysis was performed to detect GroEL and DnaK proteins in protein extract from *Synechocystis* sp. PCC6803 by using anti-*Synechococcus vulcanus* GroEL and DnaK polyclonal antibodies as probes, respectively (Roy et al., 1999). Briefly, equal amount of crude soluble proteins was separated by SDS-PAGE as described above, then electroblotted to a PVDF-membrane. After incubation with antibodies, the membrane was incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (H+L) (Gibco BRL) for detections for GroEL and DnaK.

Viability assays

Synechocystis sp. PCC 6803 cells were grown photoautotrophically at 30°C up to an OD of around 0.5 under a light intensity of 35 $\mu E/m^2/s$ as described above and then subjected to different treatments in glass tubes containing 25 ml of culture with continuous aeration. All the samples were adjusted to an OD of 0.1 at 730 nm and diluted 1:5, 1:25, 1:125, 1:625, 1:3125, and 1:15625 times in sterile BG 11 medium. A 5-μl aliquot from each dilution was spotted in triplicates on BG-11 plates for each treatment and the average of these three counts was considered as the total count. Plates were incubated at 30°C under a light intensity of 35 $\mu E/m^2/s$ for one to two weeks. Viability assays were repeated three times with independent cultures.

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Table1. Sequences of oligonucleotide primers used for probe preparation.

mRNA to be detected	Primer name	Primer sequence (5' to 3')
htpG	HtpG-9	GATTGGTCACTTTGGTTTGGGT
	HtpG-10	GATATTCTGCCGGATTTTCGTC
groES	groES-1	GACCTTGCAAGACTATCATCTG
	groES-2	GTTAACAGAACATAGTCGTCGC
groEL2	CPN-1	ATCGGCGTAAAGCTGTGTTGCA
	CPN-2	CGACTTTAATCACAGCTACACC
hsp17	shsp-1	TTCTTTACAATCCCCTGCGG
	shsp-2[a]	TAATACGACTCACTATAGGGTCA-AAGTTAGGATACCGGCAT

a. The antisense oligonucleotide primer contains the T7 RNA polymerase promoter sequence (the underlined part of the sequence).

Figure legends

Fig.1. Northern blot analysis of the HSP mRNAs accumulated in *Synechocystis* sp. PCC 6803 (A) and *Synechococcus* sp. PCC 7942 (B) under various thermal and light conditions. RNA was isolated from cells which were incubated at 30°C for 3 h in the light (35 $\mu E/m^2/s$) (30L) or dark (30D), and shifted to either 42°C for 15 min (in the case of *Synechocystis* sp. PCC 6803) or 45°C for 20 min (in the case of *Synechococcus* sp. PCC 7942) in the light (42L or 45L) or dark (42D or 45D). The results shown are representative from three independent experiments, each of which gave similar results.

Fig.2. Time course for the accumulation of different HSP mRNAs of *Synechocystis* sp. PCC 6803 after heat shock at 42°C in the presence (o) or absence (x) of light. Cells growing at 30°C under a light intensity of 35 $\mu E/m^2/s$ were subjected to heat shock in the presence or absence of light for 3 h and samples were collected at 10, 20, 30, 60, 90, 120, and 180 min intervals. Figure shows both the bands for the *htpG* (A), *hsp17* (B), *groESL1* (C), and *groEL2* (D) mRNAs detected by Northern blot analysis and the relative mRNA level quantified by Scion Image. The results shown are representative from three independent experiments, each of which gave similar results.

Fig. 3. Effect of the photosynthetic electron transport inhibitors DCMU and DBMIB on the transcriptional activation of the heat shock genes under heat stress. A culture of *Synechocystis* sp. PCC 6803 cells growing at 30°C was divided into 4 portions. One portion was used for the 30°C control. To two portions of the culture, either DCMU or DBMIB was added to a concentration of 15 μM or 10 μM, respectively. To the other portion, ethanol (EtOH) at the same volume of the inhibitors was added. These three cultures were incubated at 30°C for 5 min, and then shifted to 42°C for 15 min under a light intensity of 35 $\mu E/m^2/s$. The results shown are representative from three independent experiments, each of which gave similar results.

Fig. 4. Effect of light and DCMU on the mRNA stability after 15 min of heat shock in the presence of light. A culture of *Synechocystis* sp. PCC 6803 cells growing at 30°C was shifted to 42°C for 15 min in the presence of light (35 $\mu E/m^2/s$), and divided into two portions. After the following pairs of treatments to each portion of the culture, an aliquot from each culture was collected at intervals of 5 min, 10 min, and 20 min, simultaneously. The left column. In A, one portion of the culture was incubated in the presence of light and the other one in the absence of light. In B, rifampicin (300 μg/ml) was added to the heat-shocked culture before the treatment in A. The right column. In A, one portion of the culture was incubated in the presence of 0.1% ethanol and the other one in the presence of 15 μM DCMU. In B, rifampicin (300 μg/ml) was added to the heat-shocked culture before the treatment in A. The results shown are representative from three independent experiments, each of which gave similar results.

Fig. 5. Stability of various HSP mRNAs after 60 min of heat shock in the light or dark. A culture of *Synechocystis* sp. PCC 6803 cells growing at 30°C was shifted to 42°C for 60 min in the light (35 $\mu E/m^2/s$), and divided into two portions, and then either methanol (-Rif) or rifampicin (+Rif) was added to 0.1% or 300 μg/ml, respectively. Cells from each culture were kept under the same light conditions, and collected at intervals of 30 min, 60 min, and 120 min, simultaneously. Similar experiments were performed with the same 30°C-grown culture which was shifted to 42°C in the dark. The results shown are representative from three independent experiments, each of which gave similar results.

Fig. 6. Accumulation of GroEL and DnaK in *Synechocystis* PCC 6803 cells under various thermal and light conditions. Proteins were isolated from cells which were grown at 30°C in the light (35 μE/m^2/s) (30L), and either directly shifted to 48°C for 60 min (48L) or initially shifted to 42°C for 60 min in the light (42L) and then to 48°C for 60 min in the light (42L-48L) or dark (42L-48D). Similarly, proteins were isolated from cells which were grown at 30°C in the light, and initially shifted to 42°C for 60 min in the dark (42D) and then to 48°C for 60 min in the light (42D-48L) or dark (42D-48D). Figure shows SDS-PAGE (15%) stained with Coomassie Brilliant Blue (A) and Western bolt analysis for DnaK and GroEL (B) of 1 μg protein samples in each lane. The results shown are representative from three independent experiments, each of which gave similar results.

Fig. 7. Acclimation of *Synechocystis* PCC 6803 to a lethal temperature (48°C) after pretreatment at 42°C. Plated were cells which were grown at 30°C in the light (35 μE/m^2/s) (30L), and either directly shifted to 48°C for 60 min (48L) or initially shifted to 42°C for 60 min in the light (42L) and then to 48°C for 60 min in the light (42L-48L) or dark (42L-48D). Similarly plated were cells which were grown at 30°C in the light, and initially shifted to 42°C for 60 min in the dark (42D) and then to 48°C for 60 min in the light (42D-48L) or dark (42D-48D). Figure shows one typical cell counts on plates (A) and log of the average cell survival compared to the 30°C control as 100% from three independent experiments (B).

Fig. 1. Asadulghani *et al.*

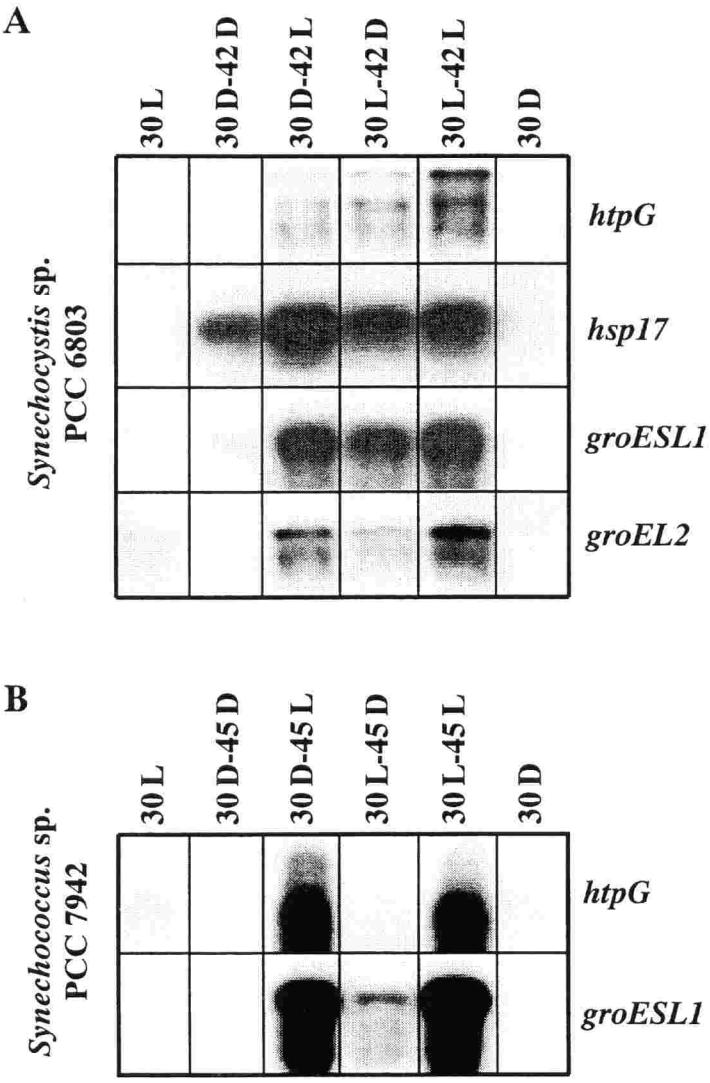
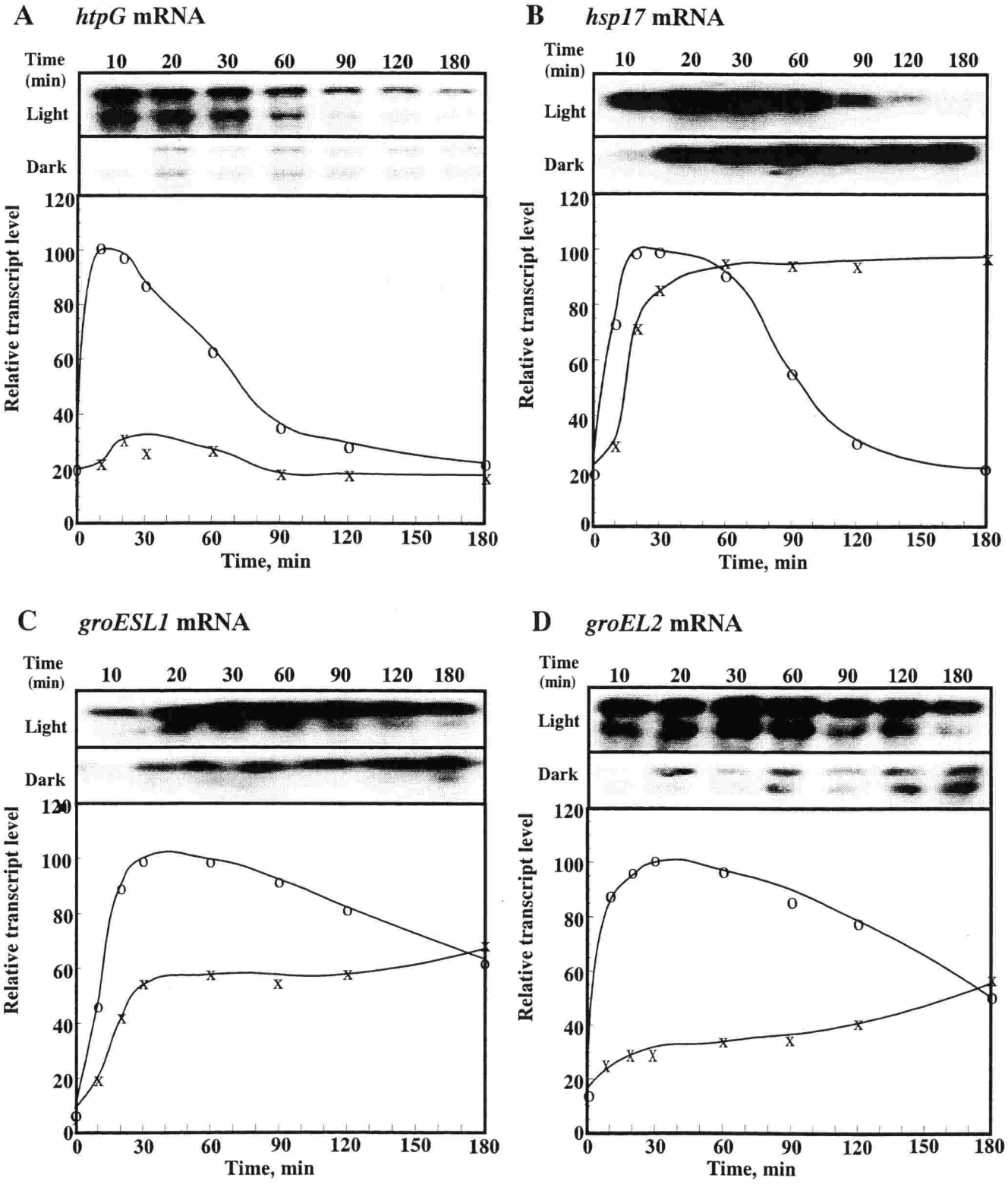


Fig. 2. Asadulghani *et al.*



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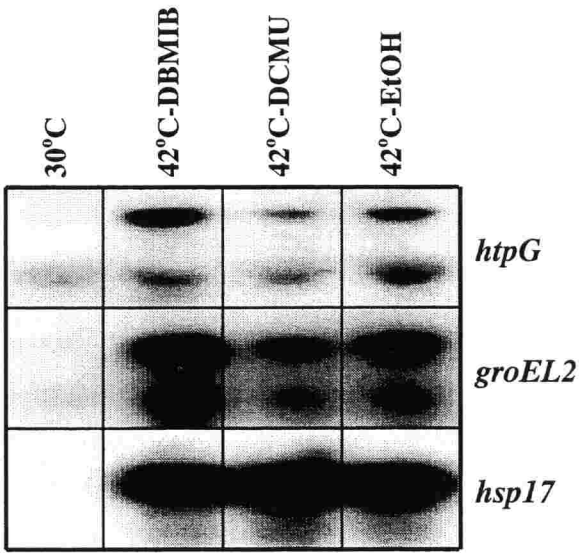
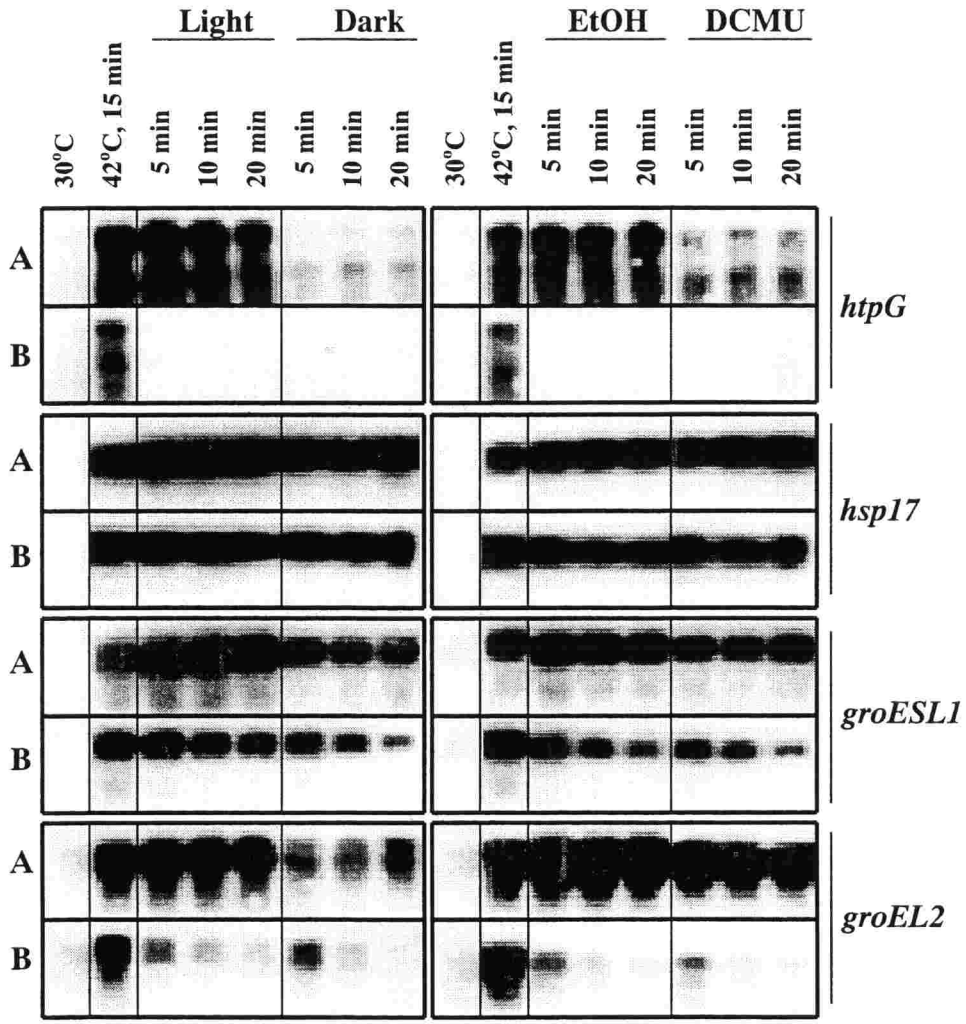


Fig. 4. Asadulghani *et al.*



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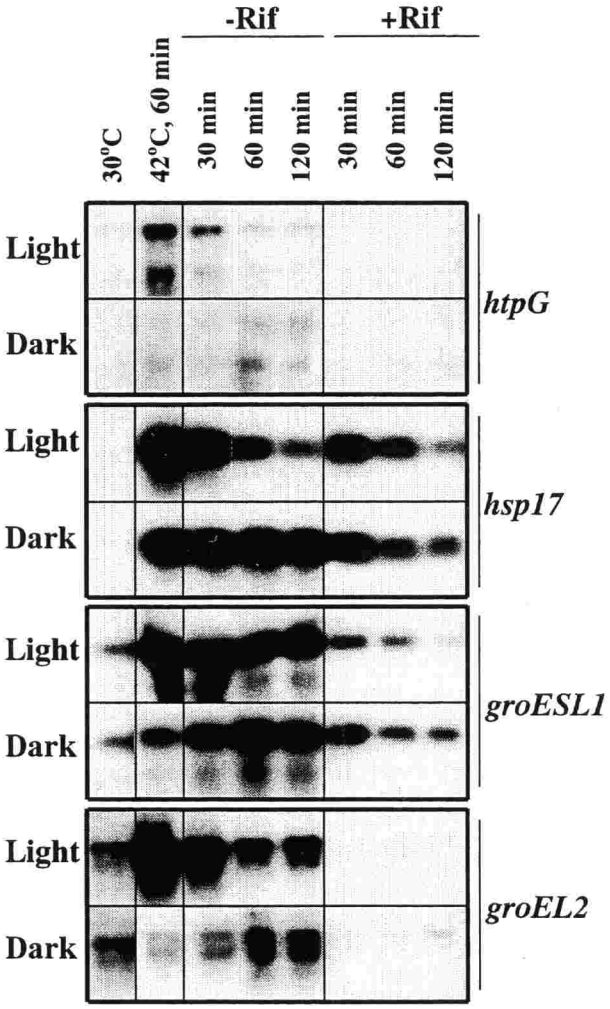

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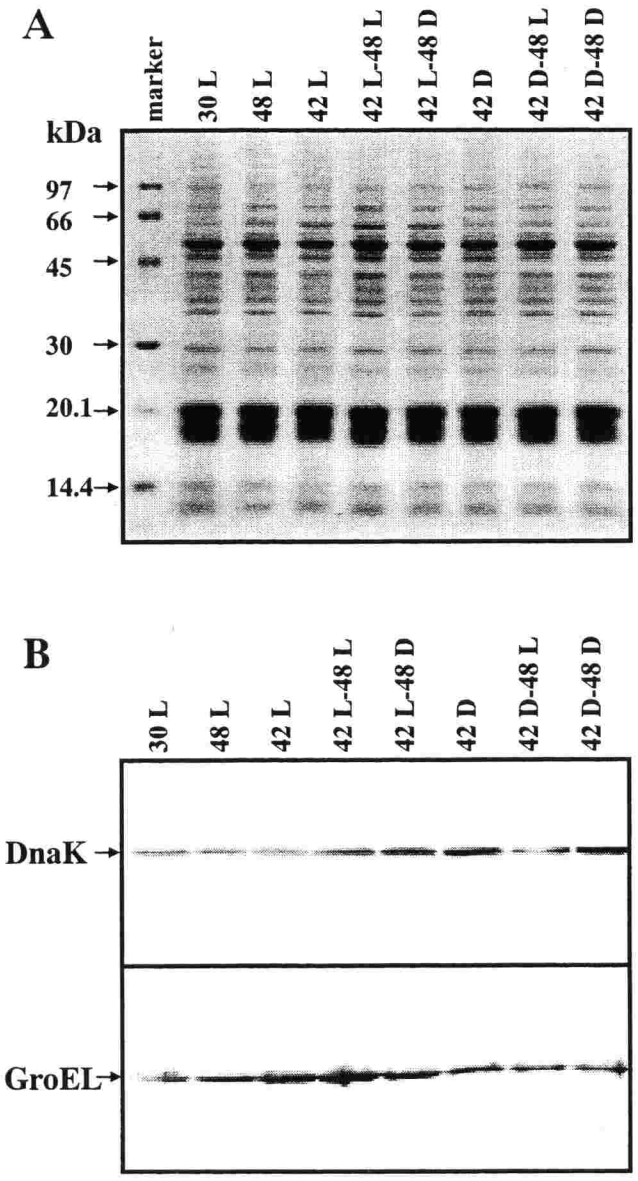

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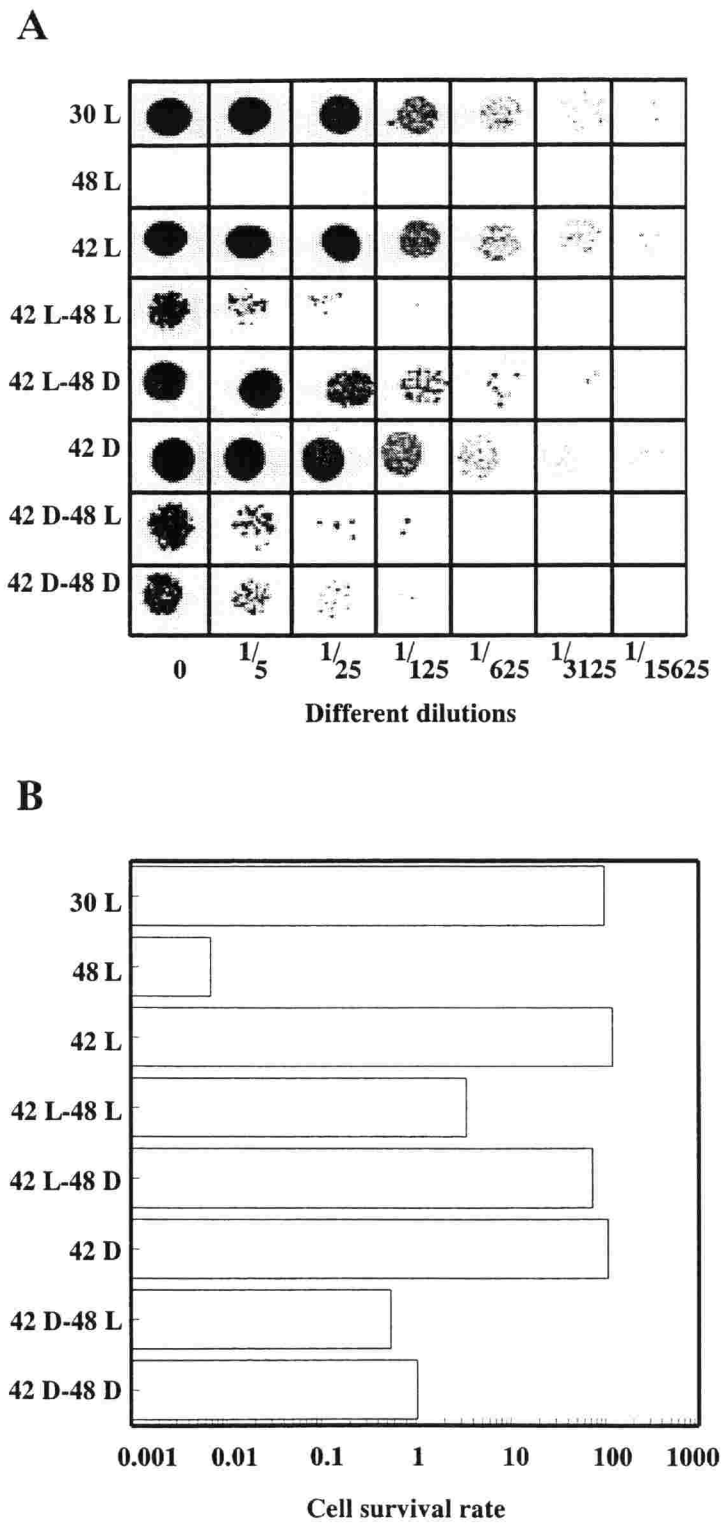

S35-023

Heat shock proteins play a role in both high and low temperature stresses in cyanobacteria

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Keywords: Cyanobacterium, Small heat shock protein, HtpG, Thermotolerance, Photoinhibition

Introduction

Cyanobacteria, like other organisms, synthesize a diverse range of heat-shock proteins (Hsps) upon exposure to high temperatures. In order to demonstrate a specific contribution of a Hsp in thermotolerance in cyanobacteria, mutants of the *clpB*, *hsp16.6* (small Hsp homolog), and *htpG* genes have been constructed by gene-targeting (Eriksson and Clarke 1996, Tanaka and Nakamoto 1999, Lee et al. 2000). Interestingly, all these disruptants showed much more striking thermosensitive phenotypes than corresponding mutants of other heterotrophic prokaryotes, suggesting that those Hsps are particularly important for photoautotrophic organisms. Here, we will show that HspA, a small Hsp homolog, and HtpG, a homolog of Hsp90, play roles in the acquisition of high and low temperature tolerances in cyanobacteria. Compared with GroEL (Hsp60 homolog) and DnaK (Hsp70 homolog), functions of small Hsp and HtpG are enigmatic. HspA from *Synechococcus vulcanus* and HtpG from *Synechococcus* sp. PCC 7942 form a homo-oligomer consisting of 24 subunits (Roy et al. 1999) and a homo-dimer (unpublished data), respectively. Both Hsps prevent the aggregation of model substrates at high temperatures (Roy et al. 1999 and unpublished data). The in vivo substrate(s) for none of the Hsps has been identified yet. Upon heat shock, small Hsp accumulates as a major Hsp in cyanobacteria (Roy et al. 1999), while HtpG appears to be a minor one (unpublished data).

Materials and methods

Organisms and culture conditions. The *Synechococcus* sp. PCC 7942 cells were cultured photoautotrophically in BG-11 inorganic medium (Tanaka and Nakamoto 1999). The liquid culture in a flat glass vessel was continuously aerated. Unless otherwise indicated, cultures were grown at 30°C with a light intensity of 30 to 40 $\mu E/m^2/s$. In order to express the *hspA* gene from *Synechococcus vulcanus* constitutively in *Synechococcus* sp. PCC 7942, it was transformed with an expression vector (pECAN8) carrying the *hspA* coding sequence, resulting in the ECT16-1 strain (Nakamoto et al. 2000). The HspA protein was overexpressed under the control of the *tac* promoter. The reference strain, ECT, was obtained by transforming the cyanobacterium with the vector containing no *hspA* gene. A knockout mutant of the *htpG* gene was constructed by insertion (Tanaka and Nakamoto 1999).

Viability assays and measurements of whole cell absorbance were performed as described previously (Nakamoto et al. 2000).

Measurements of oxygen evolution. Rates of oxygen evolution by cells were measured either in the presence of 10 mM $NaHCO_3$ for whole-chain electron transport or 1 mM 1,4-

benzoquinone and 1 mM $K_3Fe(CN)_6$ for photosystem II activity with a DW1 oxygen electrode unit (Hansatech) at 30°C at a photosynthetic photon flux density of 1,200 $\mu E/m^2/s$.

Protein extraction from Synechococcus sp. PCC 7942 cells. Cells harvested by centrifugation were suspended in 25 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, and 1 mM each of phenylmethylsulfonyl fluoride, benzamidine and caproic acid, mixed with glass beads, and disrupted by vortexing vigorously for 3 min. This process was repeated three times with a 3-min interval on ice between the vortexing, and the resulting suspension was centrifuged at 16,000 g for 30 min. The supernatant fraction was used for the non-denaturing polyacrylamide gel electrophoresis (PAGE), sodium dodecyl sulfate (SDS)-PAGE, and Western blot analysis (Roy et al. 1999).

Results and Discussion

Small Hsp confers cellular thermotolerance. The transformant (ECT16-1) which was shown to constitutively express HspA displayed improved viability compared with the reference strain (ECT) upon transfer from 30°C to 50°C in the light (Table 1). In contrast to our results, overexpression of IbpA and IbpB (small Hsp homologs) in *E. coli* led to 5- to 10-fold reductions in viability (Thomas and Baneyx 1998). When the heat shock was given in darkness, the survival rate in the reference strain increased greatly, exceeding the level for the HspA expressing strain after heat shock in the light (Table 1). These results indicate that heat stress produces a much severer effect on the cellular survival in the light than in the dark, and small Hsp plays an important role especially under the photoautotrophic conditions.

Table 1. Survival rate, photosystem II (PSII) activity, and phycocyanin level of the ECT and ECT16-1 strains after a direct shift to 50°C either in the light (30 - 40 $\mu E/m^2/s$) or in the dark.

Strain	Survival rate		PSII activity		Phycocyanin	
	Time after a shift to 50°C		Time after a shift to 50°C		Time after a shift to 50°C	
Heat shock	0	15 min	0	15 min	0	15 min
in the light						
ECT	100%	2%	100%	ND	100%	~30%
ECT16-1	100%	28%	100%	33%	100%	~80%
in the dark						
ECT	100%	32%	100%	ND	100%	~100%
ECT16-1	100%	47%	100%	9%	100%	~100%

In each heat-shock treatment shown, the value obtained before the shift to 50°C was taken as 100%. ND, not detectable. Phycocyanin level was approximated from a decrease at 625 nm in the whole cell absorbance after the shift to 50°C (see Nakamoto et al. 2000).

Identification of the possible sites of action of the small Hsp in vivo to be the photosystem II (PSII) complex and the light-harvesting phycobilisomes. In the ECT strain, PSII was heat-inactivated both in light and darkness, while the inactivation of phycocyanin took place only in the light. Expression of HspA increased thermal resistance of both PSII and phycocyanin (Table 1). Although PSII was completely inactivated in darkness as well as in the light, the survival rate increased markedly in darkness as shown above. Thus, PSII inactivation may not be the primary cause for the cell death at elevated temperatures. We propose that the

phycobilisomes may interact with PSI when PSII is inactivated. The PSI with the light-harvesting apparatus produces ATP through the cyclic electron flow, which may be used to restore PSII and other functions, and thus contribute for the increase in survival rate.

Small Hsp plays a role in the protection of the physical order of thylakoids under high temperatures. The ECT and ECT16-1 cells after exposure at 50°C for 15 min were examined by transmission electron microscopy. Significant differences were observed in cell ultrastructure between these strains. While the integrity of thylakoid membranes was disrupted in heat-shocked ECT cells, concentric layers of thylakoids at the periphery of cells were still observed in heat-shocked ECT16-1 cells (data not shown). Thus, constitutive expression of the HspA protein increased the physical order of thylakoid membranes, thus stability at elevated temperatures. The association of HspA with thylakoid membranes may serve as a membrane protection mechanism. Experiments such as immunogold localization of HspA using transmission electron microscopy are in progress.

HtpG is essential for the thermal stress management. We inactivated the *htpG* gene in the cyanobacterium, *Synechococcus* sp. PCC 7942 by gene-targeting to elucidate the role of HtpG *in vivo* (Tanaka and Nakamoto 1999). The mutant cells lost both basal and acquired thermotolerances, indicating that HtpG plays an essential role for the thermotolerance in cyanobacteria. These results with *Synechococcus* are in contract to those with *E. coli* (Bardwell and Craig 1988, Thomas and Baneyx 1998) and *B. subtilis* (Versteeg et al. 1999). Inactivation of the *htpG* gene in these heterotrophic organisms did not cause a fatal effect on cells at elevated temperatures. The photosynthetic oxygen evolution by whole-chain electron transport in the mutant was more sensitive to high temperatures than that in the wild type (data not shown), indicating that the photosynthetic apparatus may be one of the targets which HtpG may interact with.

HtpG plays a role in the acclimation to low temperatures. The inactivation of the *htpG* gene resulted in severe inhibitions of cell growth and the photosynthetic activity when the *htpG* mutant was shifted to 16°C from 30°C (data not shown). After the shift, the photosynthetic activity of the mutant continued to decrease with only negligible activity remaining after 3 days at 16°C, while the wild type kept approximately 20% of the level at 30°C even after 5 days at 16°C. The wild-type cells were able to resume growth without a lag period when shifted to 30°C after 5 days at 16°C, while the mutant displayed a detectable lag (data not shown). Electrophoresis in the absence of SDS showed that a novel high-molecular-weight (450-kDa) complex containing a 48-kDa polypeptide, GroEL, and DnaK accumulated in the wild-type cells grown at 16°C for 5 days, but the accumulation was strongly inhibited in the *htpG* mutant (Fig.1). In *E. coli*, GroEL is an 800 kDa assembly, a tetradecamer of 57 kDa subunits arranged in two stacked seven-membered rings. In cyanobacteria, GroEL may also be assembled into a tetradecamer (Lehel et al. 1992). Thus, it is unexpected that the GroEL protein exists in the 450-kDa complex. GroES was not detected immunochemically in this complex (data not shown). Since the amount of the 450-kDa protein complex was reduced in the mutant cells, the HtpG protein may be involved in the cold-induced assembly of this novel multiprotein complex. The complex may play a role in the cold acclimation. Under low temperatures, the assembly of the GroEL tetradecamer and further binding of the GroES heptamer to GroEL may be inhibited. Instead, the GroEL protein may form a novel multiprotein complex with DnaK and the 48-kDa protein.

Constitutive expression of small Hsp in the htpG mutant. The *htpG* mutant in which the HspA protein was constitutively expressed was constructed in the same manner as the construction of ECT16-1. In vitro studies have shown that small Hsp and HtpG function similarly as molecular chaperones. They keep a heat-denatured model substrate in a soluble folding-competent state which may be refolded by an ATP-dependent molecular chaperone machine such as GroES/GroEL or DnaK/DnaJ/GrpE. Thus, we thought that the thermolabile

htpG mutant may be complemented with a return to the wild-type phenotype by the constitutive overexpression of the small Hsp. However, it did not alleviate the inhibition of growth at 45°C (see Tanaka and Nakamoto 1999) in the *htpG* mutant (data not shown).

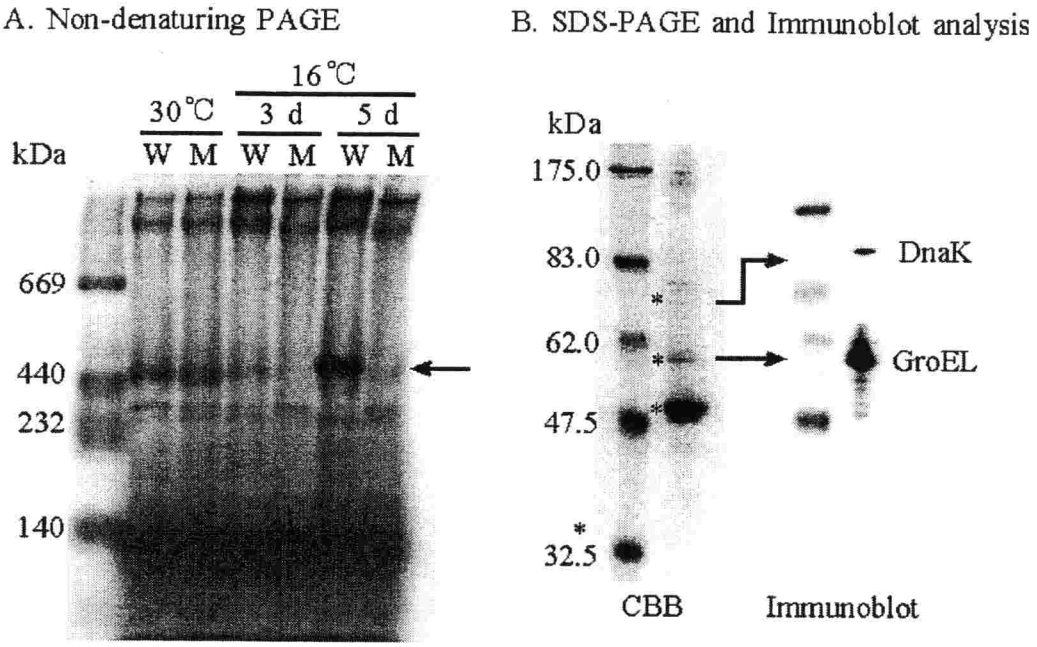

Fig. 1. Detection of a 450-kDa protein complex (indicated by the arrow) by non-denaturing PAGE (A) and detection of its components by SDS-PAGE and immunoblot analysis (B). In A, equal amounts of crude soluble proteins extracted from the wild type (indicated by 'W') or the *htpG* mutant (indicated by 'M') cells grown at 30°C, and then shifted down to 16°C for 3 days, or 5 days were separated by PAGE containing no SDS and the gel was stained by coomassie brilliant blue (CBB). In B, a CBB-stained gel strip containing the 450-kDa band indicated by the arrow in A was excised from the non-denaturing gel, applied to a sample well of a SDS-polyacrylamide gel and electrophoresed. Then, the gel was either stained by CBB, or the GroEL and DnaK proteins were specifically detected by immunoblot analysis using polyclonal antibodies raised against *Synechococcus vulcanus* GroEL and DnaK. The CBB-stained bands corresponding to DnaK, GroEL, and a 48-kDa polypeptide are indicated by asterisks.

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