Cloning and Characterization of Two groESL Operons of *Rhodobacter sphaeroides*: Transcriptional Regulation of the Heat-Induced groESL Operon

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The nonsulfur purple bacterium *Rhodobacter sphaeroides* was found to contain two groESL operons. The groESL1 heat shock operon was cloned from a genomic library, and a 2.8-kb DNA fragment was sequenced and found to contain the groES and groEL genes. The deduced amino acid sequences of GroEL1 (cpn60) and GroES1 (cpn10) were in agreement with N-terminal sequences previously obtained for the isolated proteins (K. C. Terlesky and F. R. Tabita, Biochemistry 30:8181–8186, 1991). These sequences show a high degree of similarity to groESL genes isolated from other bacteria. Northern analysis indicated that the groESL1 genes were expressed as part of a 2.2-kb polycistronic transcript that is induced 13-fold after heat shock. Transcript size was not affected by heat shock; however, the amount of transcript was induced to its greatest extent 15 to 30 min after a 40°C heat shock, from an initial temperature of 28°C, and remained elevated up to 120 min. The *R. sphaeroides* groESL1 operon contains a putative hairpin loop at the start of the transcript that is present in other bacterial heat shock genes. Primer extension of the message showed that the transcription start site is at the start of this conserved hairpin loop. In this region were also found putative −35 and −10 sequences that are conserved upstream from other bacterial heat shock genes. Transcription of the groESL1 genes was unexpectedly low under photoautotrophic growth conditions. Thus far, it has been not possible to construct a groESL1 deletion strain, perhaps indicating that these genes are essential for growth. A second operon (groESL2) was also cloned from *R. sphaeroides*, using a groEL1 gene fragment as a probe; however, no transcript was observed for this operon under several different growth conditions. A groESL2 deletion strain was constructed, but there was no detectable change in the phenotype of this strain compared to the parental strain.

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operon (10, 31). While the physiological role of multiple chaperonin genes has not been completely elucidated, a study of the groE operons in B. japonicum has indicated that one of these (groESL) is specifically expressed under conditions in which the nitrogen fixation genes are induced (10).

To further our study of chaperonin-mediated RubisCO folding and the regulation of chaperonin synthesis (38), we have isolated two distinct groESL operons from R. sphaeroides. (A preliminary report of this study appeared recently [24]).

MATERIALS AND METHODS

Materials. Sequence and TaqMan were obtained from U.S. Biochemical, Cleveland, Ohio. Restriction enzymes, DNA polymerase I, and T4 DNA ligase were from Gibco Bethesda Research Laboratories, Gaithersburg, Md. [3H] and [35S]-labeled nucleotides were from Dupont, NEN Research Products, Boston, Mass. The Sequencher cycle sequencing kit was used for Epit仑te Technologies, Madison, Wis. The random primed labeling kit and avian myeloblastosis virus reverse transcriptase were from Boehringer Mannheim, Indianapolis, Ind. Plasmid pBR322 (10), containing B. japonicum groESL, was a gift from H. M. Fisher. Sequence analysis and comparisons were performed with the Wisconsin Sequence Analysis package from the Genetics Computer Group, Madison, Wis.; PC/Genes from IntelliGenetics, Inc., Mountain View, Calif.; and the MacVector and AssemblyLIGN software programs from Kodak Scientific Imaging Systems, New Haven, Conn.

Bacterial strains and plasmids. R. sphaeroides HR is a derivative of ATCC 17023 but is capable of growth at 42°C (44). The media and growth conditions used were as previously described (38). The plasmid vectors and constructs used in this study and their relevant characteristics are listed in Table 1.

Isolation of DNA fragments. Southern hybridization and colony blotting of R. sphaeroides genomic DNA, using the B. japonicum groESL DNA as a probe, were done with 50% formamide/4× SSC (1× SSC is 150 mM NaCl plus 15 mM sodium citrate)–20 mM Tris-HCl (pH 7.6)–5% dextran sulfate–0.1% sodium dodecyl sulfate (SDS) at 40°C and washing in 2× SSC–1% SDS at 45°C. Southern hybridization to detect additional groE genes of the R. sphaeroides genome was performed with the 1.0-kb SphI-Smal restriction fragment of the heat-induced groEL gene. These Southern blots, and subsequent colony blots of cloned DNA fragments, were hybridized in 6× SSC–2× Denhardt solution (0.01% Ficoll, 0.01% polyvinylpyrrolidone, 0.01% bovine serum albumin) containing 0.1% SDS and 100 μg of denatured salmon sperm DNA per ml. The blots were then washed at 60°C overnight and then washed three or four times in 5× SSC at 60°C.

DNA sequencing. DNA sequencing was performed by the dideoxy-chain termination method (33) to sequence double-stranded DNA templates, with Sequenase. Plasmid DNA was obtained from standard alkaline lysis preparations (32). To resolve compressions due to the high G-C content of R. sphaeroides DNA, both deca-dGTP and dITP were used. In addition, some sequence data was obtained by using either TaqMan or the Sequitherm kit. The entire sequence was read on both strands to ensure that all compressions were resolved. Calculation of the free energy of hydrolysis of putative hairpin loop structures was determined with the HAIRPIN program of PC/Gene.

RNA isolation and analysis. Total cellular RNA was isolated from cells (50 ml per preparation) grown to mid-log phase (A600 of 0.6 to 0.8). The cells were transferred to 0.1 volume of stop solution (50 mM Tris-HCl [pH 8.0], 20 mM EDTA [pH 8.0], 20 mM NaCl) containing rifampin (1.5 mg/ml) and centrifuged at 3,200 × g for 10 min. The cell pellet was resuspended in 2 ml of boiling lysis buffer (100 mM Tris-HCl [pH 8.0], 2 mM EDTA [pH 8.0], 2% SDS) and kept in a boiling water bath for 5 min, after which 35 μl of 4 M potassium acetate was added. This was followed by a 5-min incubation on ice. The cell suspension was then centrifuged in a microcentrifuge for 10 min; the resulting supernatant was phenol extracted once and ethanol precipitated. The precipitated RNA samples were subsequently resuspended in 3 ml of water to which 1.5 g of cesium chloride was added. The RNA-cesium chloride solution was then layered over a 1.5-mL cushion of 5.7 M cesium chloride in an ultracentrifuge tube. The samples were centrifuged at 34,000 rpm for 19 h at 15°C in a Beckman SW50.1 rotor. The precipitate was then resuspended in water and ethanol precipitated. After re-suspension of the RNA in water, the samples were used for Northern analysis (32) and primer extension analysis. Quantitation of bands on Northern blots was performed with an Instant Imager (Packard, Meriden, Conn.). Northern hybridizations were standardized by using a 16S rRNA gene probe as a control. This probe was prepared from genomic DNA after amplification of the specific DNA by PCR with 5′ and 3′ oligonucleotides based on the reported sequence (5). The amplified DNA was subsequently cloned into plasmid pK18 and used for the 16S rRNA probe. Primer extension analysis was performed by a modification of the method of Triezenberg (40). A 19-mer oligonucleotide (5′ ACGCGGTTTG 3′) complementary to the +535 to +553 region of the R. sphaeroides groESL coding sequence, was kinase treated with 50 μCi of [γ-32P]ATP, ethanol precipitated three times, and then hybridized to 100 to 200 ng of total R. sphaeroides RNA. The primer extension reaction was performed with avian myeloblastosis virus reverse transcriptase.

Deletion mutagenesis of groESL. Digestion of pUCG2BS with SalI and PstI removes a 1.62-kb fragment within the groESL genes. The blunt-ended HindIII kanamycin resistance cassette from pUC18Km was inserted into the blunt-ended SalI-PstI site. A 4.2-kb Km fragment containing the deletion construct was ligated into pJP5603. Selection for the R. sphaeroides groESL deletion strain was made by mating pJP5603 with E. coli C571-λ pprox into R. sphaeroides HR on noninvasive PYE (44) plates and then plating cell mixtures from overnight incubations onto Omerod’s minimal malate plates containing kanamycin. Double recombinants were scored as those Km transconjugants that were Sp+, indicating the loss of plasmid vector sequences (29). Southern analysis verified that the expected homologous recombination had taken place.

Nucleotide sequence accession numbers. The nucleotide sequence of the R. sphaeroides groESL operon has been deposited in the GenBank library under accession number U67369; the nucleotide sequence of the groESL operon is under accession number U66831.

RESULTS

Cloning and sequencing of the groESL operons. Southern blot of restriction enzyme digests of R. sphaeroides genomic DNA probed with a 1.7-kb ApaI-BstI fragment of B. japonicum groESL operon, exhibited a single SalI restriction fragment of approximately 8.0 kb. SalI-digested genomic DNA, ranging between 6 and 9 kb, was isolated and ligated to plasmid pK18, and a plasmid containing an 8.0-kb SalI insert was isolated after screening of this partial library with the B. japonicum groESL probe. The approximate location of the groESL genes within the 8-kb restriction fragment was determined by Southern analysis; subsequent sequencing verified that the R. sphaeroides groESL operon had been cloned. The R. sphaeroides groESL1 operon was localized to a 2.8-kb restriction fragment (Fig. 1A) and subsequently completely sequenced with additional plasmids and primers. The DNA sequence (Fig. 1B) of the groESL1 operon revealed two open reading frames with a high degree of similarity to other bacterial groESL operons.

Southern blots of R. sphaeroides genomic DNA, probed with a 1.0-kb SphI-Smal groESL1 fragment, showed the presence of a second putative groESL operon (Fig. 2). A 2.3-kb KpnI restriction fragment was isolated from a library of R. sphaeroides genomic DNA. Partial DNA sequencing of this restriction fragment indicated that it contained most of a second groEL

| Table 1. Plasmids used in this study |
|-------------------------------------|
| Plasmid(s) | Relevant characteristics | Source or reference |
| pK18, pK19 | Km', pUC derivatives | 30 |
| pUC18 | Am' | 46 |
| pKTS0 | pK18 containing 8.0-kb SalI fragment | This study |
| pG2BB | pK18 containing 7.0-kb BamHI fragment containing entire groEL operon | This study |
| pG2BBlam | pK18 containing 4.0-kb BamHI-BglII fragment of pG2BB | This study |
| pUCG2 | pUC18 containing 4.0-kb insert of pG2BBlam as EcoRI-HindIII fragment | This study |
| pUCG2ΔS | pUCG2 digested with Sph-XbaI (in polylinker) and filled in | This study |
| pUC1318K | pUC1318 with kanamycin resistance gene | 7 |
| pG2ΔKn | pUCG2ΔS lacking 1.62-kb SalI-PstI fragment with Km' gene cartridge inserted | This study |
| pJP5603Ω | pJP5603 containing 8 fragment | H. Xu; this laboratory |
| pJPΔ2ΔKn | pJP603Ω with 4.4-kb KpmI fragment of pG2ΔKn inserted | This study |
gene, as well as some 3′ untranslated sequence. This KpnI restriction fragment was used to isolate a 7.0-kb BamHI restriction fragment that encoded the entire operon (Fig. 3A). Sequencing indicated the presence of both groES and groEL genes (Fig. 3B). However, it appears that the groES2 gene is a pseudogene. There is homology to the groES1 gene product in two of the groES2 reading frames (Fig. 3B), and the codon usage is very poor for R. sphaeroides. In addition, the deduced

FIG. 1. Restriction map and nucleotide sequence of the R. sphaeroides groESL1 operon. (A) Restriction map of the heat induced groESL1 operon. The 2.8-kbp fragment was sequenced on both strands by using subclones and oligonucleotides. Abbreviations: B, BamHI; Bg, BglII; P, PstI; Sm, SmaI; Sp, SphI; Ss, SstI. (B) Nucleotide sequence of the R. sphaeroides groESL1 operon. The deduced amino acid sequences of the cpn10 and cpn60 proteins are listed below the DNA sequence. The underlined sequences represent potential -10 and -35 promoter sequences; the putative hairpin loop structures are denoted by the arrows.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{Restriction map and nucleotide sequence of the R. sphaeroides groESL1 operon.}
\end{figure}
other known conserved sequences in that it contains a G instead of an A at the third nucleotide on the 5' branch of the hairpin (Fig. 4A). The G-T base pairing of the \textit{R. sphaeroides} hairpin structure results in a 4-kcal/mol difference in the free energy of formation of this structure compared with bacterial hairpins that retain the conserved A-T base pairing. There are also conserved $-10$ and $-35$ upstream sequences, which are also found associated with several other bacterial \textit{groESL} genes (Fig. 4B). Primer extension studies indicated that the transcript begins at the 5' end of the conserved hairpin loop (Fig. 4C). A minor transcription start site is also seen 3' to the hairpin loop. It is plausible that the upstream promoter region may have homology to both $\sigma^{70}$ and $\sigma^{32}$ consensus sequences (Fig. 4D).

**Comparison of chaperonin amino acid sequences.** The deduced amino acid sequence of \textit{R. sphaeroides} cpn10 and cpn60 shows considerable similarity to sequences previously reported from other organisms. Alignment of cpn60 sequences from all divisions of eubacteria, as well as eucaryotic mitochondria and chloroplasts (data not shown), was in agreement with an alignment previously published (17), with \textit{R. sphaeroides} cpn60 most closely related to cpn60s from other members of the alpha subdivision of purple bacteria. Finally, the deduced sequences of the two proteins of the \textit{R. sphaeroides} groEL genes showed 72.5% identity.

**Transcription of the groESL operon.** Northern analysis of total RNA from photoheterotrophically cultured cells indicated that a 2.2-kb \textit{groESL}$_1$ transcript was present that did not change in size after heat shock (cells were grown at 28°C and transferred to a 40°C illuminated water bath). However, levels of \textit{groESL}$_1$-specific mRNA were enhanced 13-fold upon heat shock (Fig. 5), with maximum increases occurring between 15 and 30 min after heat shock. Changes in 16S rRNA transcripts were not substantial during heat shock; thus, this parameter provides an excellent internal standard in these experiments. The results indicated that transcript levels remained 10-fold elevated up to 120 min of heat shock; however, in other experiments there was a slight variance in \textit{groESL}$_1$ transcripts from 60 to 120 min of heat shock. The \textit{groESL}$_1$ operon was also induced by heat shock when cells were cultured under photoheterotrophic and chemoheterotrophic growth conditions (data not shown). There was four times as much \textit{groESL}$_1$ transcript in photoheterotrophically grown cells as in cells grown under photoautotrophic conditions (Fig. 6). The levels of 16S rRNA were very similar in these cells (data not shown).

**Transcription of the cbbL operon.** Northern analysis of RNA from cells grown photolithoautotrophically and photoheterotrophically at 28°C and heat shocked at 40°C. The \textit{cbbL}$_1$ gene product has a calculated molecular weight of 56,789 and a pI of 5.07 and uses the typical amino acid codons for \textit{R. sphaeroides}. Use of either \textit{R. sphaeroides} heat shock \textit{groEL}$_1$ (1.0-kb \textit{Sall-SphI} fragment) or \textit{groEL}$_2$ (900-bp \textit{SphI-PstI} fragment) as a probe gave no indication of additional \textit{groEL} sequences (Fig. 2).

The deduced amino acid sequence indicated that \textit{groES}$_1$ encodes a protein of 95 amino acids with a calculated molecular weight of 10,196 and a pI of 5.24. The \textit{groEL}$_1$ gene encodes a protein of 547 amino acids with a calculated molecular weight of 57,946 and a pI of 4.81. The \textit{groES}$_1$ and \textit{groEL}$_1$ genes were expressed in \textit{E. coli} and yielded high levels of recombinant protein (results not shown). There are 96 bp between the stop codon of \textit{groES}$_1$ and the start codon of the \textit{groEL}$_1$ gene. Three potential stem-loop structures were identified which may play a role in the regulation of transcription. A putative hairpin loop structure 5' to the start site of the \textit{groES}$_1$ gene has an estimated free energy of formation of $-8.2$ kcal (1 kcal $= 4.184$ kJ/mol) (Fig. 4A). This hairpin loop is conserved in many other bacterial heat shock genes. A putative hairpin or stem-loop structure, beginning 27 bp past the stop codon of \textit{groEL}$_1$, with a calculated free energy of formation of $-30.0$ kcal/mol, is followed by another potential hairpin loop (free energy of formation of $-17.2$ kcal/mol). The two hairpin loop structures located 3' to the \textit{groEL}$_1$ gene may be involved in transcription termination, although no further studies have been performed to substantiate this.

The upstream \textit{R. sphaeroides} hairpin sequence differs from...
FIG. 3. Restriction map and nucleotide sequence of the \textit{R. sphaeroides} \textit{groESL} operon. (A) Restriction map of the \textit{groESL} operon. Abbreviations are the same as in Fig. 1A and as follows: \textit{K}, KpnI, \textit{Sl}, SalI. (B) Sequence of the \textit{groESL} operon. The deduced amino acid sequences of the putative cpn10 and cpn60 proteins are listed below the DNA sequence. The \textit{groESL} gene has homology to the \textit{groES} gene product in two of its reading frames. The regions with homology to the \textit{groES} gene product are underlined.
rich and minimal media, and no colonies containing a double recombination knockout of the groESL₁ genes were isolated. More than 2,000 colonies were screened in an attempt to isolate a deletion of the groESL₁ operon. These results may indicate that the groESL₁ genes are essential for growth in R. sphaeroides. On the other hand, the groESL₂ genes were successfully deleted and the constructed strain grew identically to the parental strain under photoautotrophic, photoheterotrophic...
DISCUSSION

Nonsulfur purple bacteria catalyze several important metabolic processes, including nitrogen fixation (23, 26), hydrogen uptake (41), and autotrophic carbon dioxide assimilation (37). In each case, large quantities of biosynthetic energy must be expended to meet the requirement for the substantial levels of proteins and enzymes that are specific to these processes. These organisms also synthesize a copious intracellular membrane network, which houses the photosynthetic apparatus, in response to the levels of oxygen present in the culture (4). In all of the above scenarios, proper folding and subsequent assembly of large amounts of newly synthesized protein are required. In view of the well-established role of chaperonin proteins in assisting the folding of unfolded polypeptides after they are synthesized (49), studies on the role of such proteins in R. sphaeroides were initiated. In particular, we are interested in how these proteins influence carbon metabolism in this organism. The cloning of two groEL operons of R. sphaeroides reported here is thus a step towards understanding the role of chaperonin proteins in nonsulfur purple bacteria. In addition to their role in protein folding, chaperonins are also known to be involved in the heat shock response, as well as other stress-related processes (49). Presumably, the interaction with unfolded proteins is maximized during times of stress; thus, the requirement for up-regulating chaperonin protein synthesis seems apparent. RubisCO, which has a central role in the metabolism of R. sphaeroides (37), has been used as a model protein to gain an understanding of how chaperonin proteins influence or assist the folding pathway (14, 15, 39). In addition, the presence of multiple cpn60/cpn10 chaperonins in different bacteria, including two of the alpha subdivision of gram-negative bacteria, provides a rationale for considering the potential of specialized chaperonins interacting with different proteins. In R. sphaeroides, it has already been demonstrated that the level of at least one cpn60 is related to growth conditions that favor enhanced RubisCO synthesis (38). Thus, it is possible that a major function of cpn60 in R. sphaeroides is to facilitate the folding and assembly of the large quantities of RubisCO and other proteins involved in carbon fixation and subsequent metabolism. Indeed, a physical association of cpn60 and form I RubisCO of R. sphaeroides has recently been described (42) and the overexpression of both the groEL1 and groES1 genes in E. coli to yield copious quantities of recombinant cpn60 and cpn10 should greatly facilitate in vitro studies.

The groEL1 genes described here represent a typical bacterial heat shock groESL operon. Like many bacterial heat shock groESL genes, groES1 and groEL1 are cotranscribed and possess a conserved upstream hairpin loop structure, as well as conserved upstream −10 and −35 sequences. The deduced amino acid sequences of cpn60 and cpn10 of R. sphaeroides agree with N-terminal sequences derived from purified proteins described earlier (38, 43) and are most closely related to similar sequences obtained from bacteria in the alpha subdivision. Within this subdivision are B. japonicum and R. melliloti, both of which possess multiple groESL operons. Other groESL genes from this subdivision have been cloned from Brucella abortus (16) and Agrobacterium tumefaciens (35). In these organisms, only a single heat shock groESL operon was cloned after screening with E. coli groESL probes. Our initial library screen with the B. japonicum groESL2 probe also identified
only a single groESL gene. However, when genomic DNA was rescreened with a probe prepared from R. sphaeroides groESL1, a second groEL gene was identified. Transcripts to the second groEL were not seen, even after heat shock. It also appears the groES gene is a pseudogene.

The transcription start of the first groEL operon was determined by primer extension. Much like that of A. tumefaciens (35) and Clostridium acetobutylicum (28), transcription begins at the 5′ end of the conserved hairpin loop. The putative −35 and −10 sequences present upstream of the R. sphaeroides groEL genes are conserved in 5′ sequences of heat shock genes from several bacteria. The hairpin loop, which also seems to be highly conserved and has been referred to as CIRCE, for controlling inverted repeat of chaperone expression (50), has been postulated to be important in the heat shock response; however, its precise function has not been determined. In this context, the CIRCE of Bacillus subtilis appears to negatively regulate transcription under nonstress conditions (50). Another study further demonstrated that CIRCE is involved in transcript turnover, as transcripts which lacked the hairpin loop were much more stable (47). A recent paper indicated that the groEL operon was still induced by heat shock, even after most of the CIRCE was deleted (36). There are CIRCE-like sequences in three of the R. sphaeroides groEL operons; however, only one of these (groEL1) is induced by heat shock (1). The CIRCE sequence has no apparent function for the other two operons. In addition, the groEL1 operon B. japonicum is induced by heat shock but lacks the CIRCE sequence (1). It should also be noted that the role of the conserved −10 and −35 sequences in the regulation of the heat-induced groEL operons containing the CIRCE sequence has not been determined. Analysis of the groESL1 upstream region of R. sphaeroides indicates that it is plausible that this operon is under the control of two RNA polymerases, one of which (σ70) is active under normal growth conditions and another (σ32) transcribes the operon during heat shock or other stress (Fig. 5D). The determination of the role of this putative promoter sequence would be of great interest if there were indeed complex regulation of this operon by different RNA polymerases.

While chaperonins have been shown to be involved in assisting the folding and, perhaps, subsequent assembly of many proteins, the interaction of cpn60 and cpn10 with Rubisco has been most extensively studied (39). Indeed, in R. sphaeroides, the levels of cpn60 were greatest under growth conditions in which Rubisco synthesis was also maximized (38). Thus, the unexpectedly lower transcription of groESL1 under photoautotrophic growth conditions might indicate that the products of groEL1 (despite our inability to demonstrate a specific transcript), or other undiscovered chaperonin proteins, are maximally synthesized when CO2 is the sole source of carbon. The lower level of transcription of the groEL1 operon may indicate that the level of cpn60 protein is regulated posttranscriptionally under photoautotrophic conditions since Southern blotting experiments indicated that there were no other groE operons. In addition, the N-terminal sequence of cpn60 isolated from two different laboratories (38, 43) agrees with the sequence of the groEL1 gene product reported here. Until a specific groESL1 transcript or specific cpn60 protein can be demonstrated, the results presented here indicate that only the groEL1 operon is expressed in R. sphaeroides, suggesting that the products of these genes play an important role in the folding and assembly of Rubisco and other proteins in this organism. Future studies will be directed at biochemical and genetic investigations of the role of each cpn60 in Rubisco folding, spurred by the isolation of a cpn60-form I Rubisco complex from extracts of R. sphaeroides (42). The recent demonstration that the intracellular levels of chaperonins influence the synthesis of proteins involved in carbon metabolism in E. coli (21) suggests that the correlation between Rubisco and cpn60 synthesis (38) may be of great importance in R. sphaeroides.

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