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# Structures of Chaperonins from an Intracellular Symbiont and Their Functional Expression in *Escherichia coli groE* Mutants

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**An intracellular symbiont harbored by the aphid bacteriocyte, a specialized fat body cell, synthesizes in vivo substantially only one protein, symbionin, which is a member of the chaperonin-60 family of molecular chaperones. Nucleotide sequence determination of the symbionin region of the endosymbiont genome revealed that it contains the two-cistron operon *sym*. Just like the *Escherichia coli groE* operon, the *sym* operon was dually led by a heat shock and an ordinary promoter sequence. According to the nucleotide sequence, symbionin was 85.8% identical to GroEL of *E. coli* at the amino acid sequence level. SymS, another protein encoded in the *sym* operon, which is a member of chaperonin-10, was 79.6% identical to GroES. Complementation experiments with *E. coli groE* mutants showed that the chaparonin-10 and chaparonin-60 genes from the endosymbiont are expressed in *E. coli* and that they can function as molecular chaperones together with endogenous GroEL and GroES, respectively.**

Prokaryotic endosymbionts harbored by the aphid bacteriocyte are inherited by the next generation at an early stage of the host's embryogenesis (2). They live only in the host cell cytoplasm, and they have no free-living stage. As a result, they cannot propagate themselves when taken out of the host cell. Aposymbiotic aphids that have lost these endosymbionts as a result of antibiotic or heat treatment are markedly undersized and sterile (14, 25). Thus, the aphid and its endosymbionts are intimately mutualistic with each other, which makes these endosymbionts unique organisms quite different from common free-living bacteria. From the nucleotide sequence data for 16S rRNA of pea aphid endosymbionts, it has been suggested that they are members of the γ subdivision of the class *Proteobacteria* and that they diverged from *Escherichia coli* about 420 million years ago (32).

The aphid endosymbiont synthesizes in vivo substantially only one protein, symbionin, with a molecular mass of 63 kDa (13). To elucidate the biological role of symbionin, we previously purified it (9) and partially determined its amino acid sequence, which revealed that symbionin is very similar to *E. coli* GroEL protein (8), a heat shock protein that is a member of the chaperonin-60 (cpn60) family of molecular chaperones (11). Cpn60 is widely distributed from bacteria to eukaryotic organelles and is involved in the folding (4), assembly (4, 5), and translocation (1, 19) of other polypeptides. Our previous study showed that symbionin is able to reconstitute dimeric ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBisCO) holoenzyme from its unfolded subunits in vitro, suggesting that this protein also functions as a molecular chaperone in the endosymbiont.

In *E. coli*, cpn60 (GroEL protein) requires GroES protein, another heat shock protein that is a member of the chaperonin-10 (cpn10) family, in order to function as molecular chaperone in the process of bacteriophage morphogenesis (30, 31). The two proteins are also essential for cell viability at all temperatures in *E. coli* (3). In contrast, no information is available about the involvement of cpn10 in the chaperonin function in vivo of organellar cpn60s such as mitochondrial hsp60 (15, 23, 26, 27) and the RuBisCO-binding protein of chloroplasts (11). Since, according to the endosymbiosis theory, the endosymbiont is supposed to be midway between a free-living bacterium and an organelle, it is intriguing to know whether symbionin, an endosymbiotic cpn60, requires cpn10 to function in vivo.

In the present study, to obtain additional information about the chaperonin function of symbionin, we isolated a DNA fragment encoding symbionin and its flanking regions and determined its nucleotide sequence. Also, using this cloned DNA from the endosymbiont, we performed complementation experiments with *groEL* and *groES* mutants of *E. coli* to assess the activity of endosymbiont chaperonins in heterologous cells.

## MATERIALS AND METHODS

**Insect materials.** A long-established parthenogenetic clone of pea aphids, *Acyrthosiphon pisum* (Harris), was maintained on young broad bean plants, *Vicia faba* (L.), at 15°C in a long-day regimen with 18 h of light and 6 h of dark.

**Bacterial strains.** *E. coli* mutant strains NRK233 (*groES619 zje*::Tn*10*) and NRK117 (*groEL44 zje*::Tn*10*), which are derivatives of MC4100 [*groE*$^+$ *araD139* Δ(*argF-lac*)*U169 rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR*], were kindly provided by K. Ito (18).

**Isolation of intracellular symbionts.** Primary endosymbionts of the pea aphid *A. pisum* were isolated essentially as described by Ishikawa (12) and Harrison et al. (10). Special care was taken not to damage the endosymbionts mechanically during homogenization of the insect materials. Living pea aphids (about 5 g) were sterilized with 70% ethanol, quickly washed twice with distilled water, and homogenized carefully in 6 volumes of ice-cold buffer A (35 mM Tris-HCl [pH 7.6], 0.25 M sucrose, 25 mM KCl, 1 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride). The homogenate was passed through a 90-μm-pore-size nylon mesh and centrifuged at 1,700 × *g* at 4°C for 20 min. The

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pellet was gently suspended in buffer A and layered over a discontinuous Percoll density gradient that contained 9, 18, 27, 36, and 45% Percoll. Percoll solutions were prepared by diluting 100% Percoll containing 1% bovine serum albumin, 5% polyethylene glycol 6000, 1% Ficoll, and 8.6% sucrose in buffer A. The gradient was centrifuged at 1,000 × *g* at 4°C for 20 min. Endosymbionts concentrated at the boundary between 36 and 45% Percoll were recovered and washed in buffer A.

**Preparation of endosymbiont DNA.** Isolated endosymbionts were incubated in lysis buffer (0.1 M Tris-HCl [pH 8.0] containing 0.1 M NaCl, 0.1 M EDTA, 1% sodium dodecyl sulfate [SDS], and 0.1% proteinase K) at 42°C for 1 h. After lysis, ordinary phenol-chloroform extraction and ethanol precipitation were performed, and the fibrous DNA was collected with a glass rod.

**DNA library construction and screening.** To isolate the symbionin gene, we first performed Southern blot analysis of isolated endosymbiont DNA using an *E. coli* DNA fragment containing the *groEL* coding region (*Eco*RV-*Eco*RV 2.1-kb fragment) derived from λ648 (17) as a probe. A 14-kb single band was detected by *Eco*RI digestion under low-stringency hybridization and washing conditions (data not shown). Then, a genomic library of endosymbiont DNA fragments of 12- to 16-kb *Eco*RI digests in a λEMBL phage vector was constructed and screened with the same *E. coli* DNA probe. The hybridization buffer contained 50% (vol/vol) formamide, 5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM $NaPO_4$, and 1 mM EDTA [pH 7.7]), 0.1% SDS, 5× Denhardt's solution, and 100 μg of denatured sperm DNA per ml, and the hybridization was performed at 38°C. The filters were washed for 30 min at 55°C with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) containing 0.1% SDS. The insert from the positive clone (*Eco*RI 14-kb fragment) was subcloned into pBluescriptKS+ (pOTKS1, shown in Fig. 3c). Fragments of this DNA were subcloned into plasmid pBluescriptKS+ or SK+, and a series of overlapping deletions of these plasmids were constructed as described by Sambrook et al. (28). The nucleotide sequence was determined by the dideoxy method (29).

**Plasmid construction and complementation tests.** pOTKS1 was constructed by inserting the 14-kb *Eco*RI fragment encompassing the *symS-symL* region into pBluescriptKS+. pOTKS2 was constructed as follows. pOTKS1 was digested with *Pst*I, and the resultant DNA fragments of 7.2 and 6.2 kb were ligated and introduced into the host *E. coli* (XL1-Blue). The direction of the ligated inserts of plasmids prepared from the transformants was determined by restriction enzyme digestion. Another DNA library of 6- to 8-kb *Hin*dIII fragments in λL47 was screened with a probe containing only the *groES* region of *E. coli* DNA to obtain the complete *symS* and incomplete *symL* regions. The inserted DNA fragments from the positive clone were subcloned into pBluescriptSK+ (pSYHD2). *E. coli* NRK117 and NRK233 were transformed by recombinant plasmids at 30°C. After being cultured at 30°C overnight, the colonies which grew were spread on two ampicillin plates. One plate was incubated at 42°C, and the other was incubated at 30°C. To examine plaque-forming ability, $2 \times 10^8$ cells of each transformant were mixed with a λEMBL phage suspension (containing about $10^3$ phage) in top agar and plated on L plates. Plates were incubated at 37°C overnight.

**Nucleotide sequence data.** The nucleotide sequence data reported here will appear in the EMBL data base under the accession number X61150.

## RESULTS

**Isolation and sequencing of a DNA clone for symbionin.** Since our previous study had indicated that symbionin is very similar to the *E. coli* GroEL protein in amino acid sequence, we used a DNA fragment from the *groE* operon to probe the symbionin gene in an endosymbiont's genomic library and detected a positive clone. After subcloning and mapping this positive insert with restriction endonucleases, we determined the nucleotide sequence of 2,739 bp containing the symbionin-coding region (Fig. 1).

It was found that the symbionin gene, designated *symL*, codes for a polypeptide of 548 amino acids which apparently belongs to the cpn60 family. An additional coding region, designated *symS*, for a polypeptide of 96 amino acids which belongs to the cpn10 family was found upstream from *symL*. Therefore, the fragment sequenced contained a two-cistron operon structure, which we named the *sym* operon.

**Characterization and homology analysis of the *sym* operon.** The promoter region of the *sym* operon was very similar to that of the *groE* operon of *E. coli*, in which a heat shock and an ordinary promoter sequence are adjacent. Around 10 bp upstream of *symS* was a Shine-Dalgarno sequence. *symL* was separated from *symS* by a 45-bp noncoding region, in which another Shine-Dalgarno sequence was detected. Around 50 bp downstream from the end of the *symL* coding sequence was a sequence which may assume the stem-loop structure that terminates the transcription of the *sym* operon (Fig. 1).

Homology analysis (Fig. 2) revealed that SymL (symbionin) and SymS from the endosymbiont are 85.8 and 79.6% identical to GroEL and GroES of *E. coli*, respectively, at the amino acid sequence level, indicating that these endosymbiont chaperonins are the most identical to those of *E. coli* among the chaperonins sequenced so far (7).

**Rescue of *E. coli groE* mutants with *symL* and *symS* expression plasmids.** To examine whether SymL and SymS are expressed and function in *E. coli*, we performed complementation tests with *E. coli groE* mutants (Fig. 3). Two mutants carrying *groES619* (NRK233) and *groEL44* (NRK117) were used (18). These mutants exhibit temperature-sensitive cell growth and do not support growth of bacteriophage λ (30).

Each strain was transformed with pOTKS2, pSYHD2, or pBluescript, and complementation of the phenotypes of the mutants was examined (see Materials and Methods). The *groEL*(Ts) mutant, NRK117, regained viability at 42°C and formed plaques with λ phage when transformed with plasmid pOTKS2 expressing SymL alone. Similarly, the *groES*(Ts) mutant, NRK233, was complemented with plasmid pSYHD2 containing the entire *symS* sequence but not complete *symL*. Neither NRK117 nor NRK233 was rescued by the control plasmid pBluescriptKS+ (Fig. 3a and b). The results indicated that the heterologous chaperonin combinations of symbionin (cpn60 of the endosymbiont) and GroES (cpn10 of *E. coli*) and GroEL (cpn60 of *E. coli*) and SymS (cpn10 of the endosymbiont) are functional as molecular chaperones in *E. coli*.

## DISCUSSION

The nucleotide sequence determination of the symbionin-coding region revealed that it contains a two-cistron *sym* operon. The *sym* operon is led by heat shock and ordinary promoter sequences, which are arranged adjacently. This finding suggests that in the transcriptional initiation of the

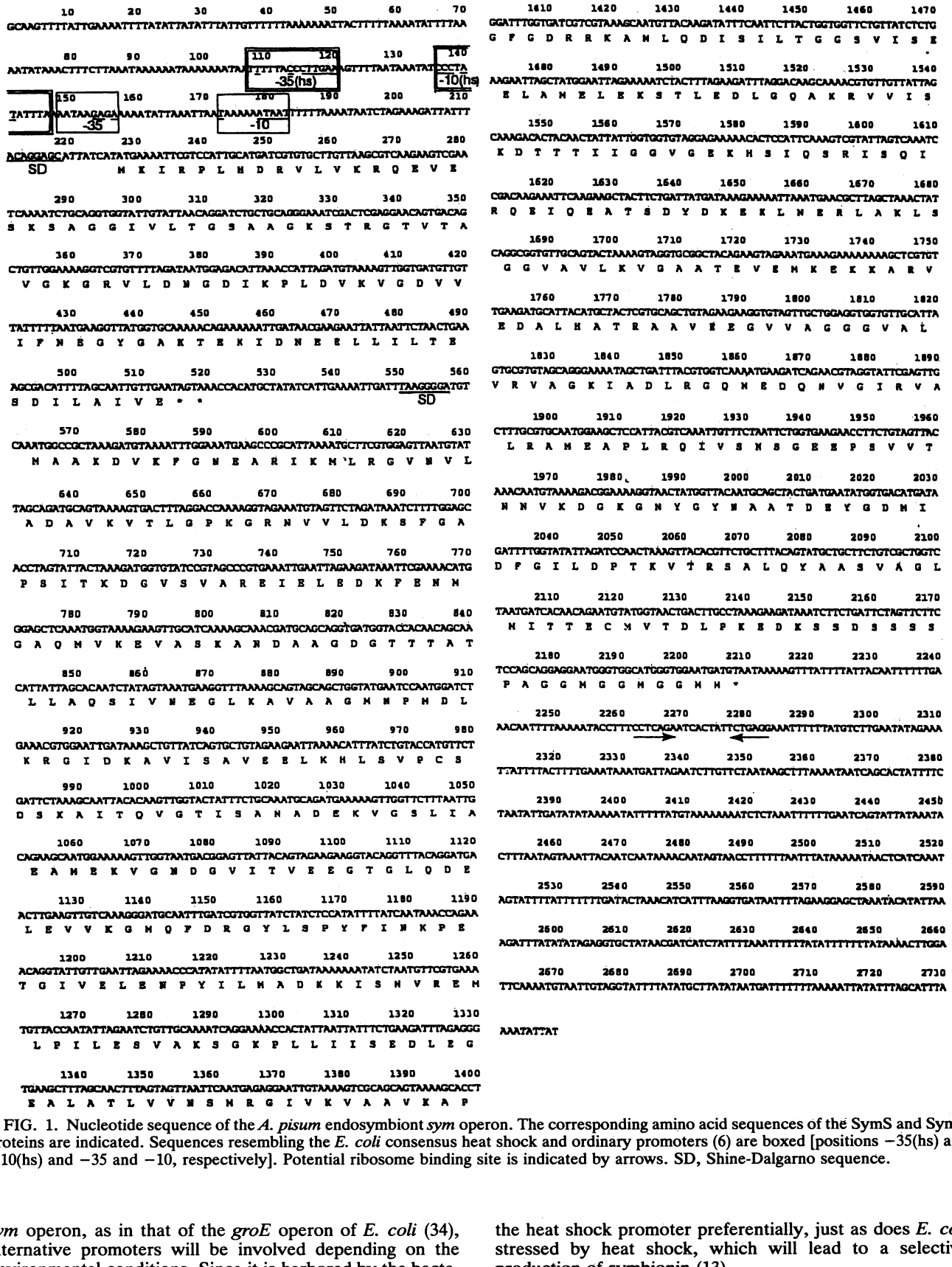


FIG. 1. Nucleotide sequence of the *A. pisum* endosymbiont *sym* operon. The corresponding amino acid sequences of the SymS and SymL proteins are indicated. Sequences resembling the *E. coli* consensus heat shock and ordinary promoters (6) are boxed [positions −35(hs) and −10(hs) and −35 and −10, respectively]. Potential ribosome binding site is indicated by arrows. SD, Shine-Dalgarno sequence.

*sym* operon, as in that of the *groE* operon of *E. coli* (34), alternative promoters will be involved depending on the environmental conditions. Since it is harbored by the bacteriocyte, the aphid endosymbiont may be constrained to use the heat shock promoter preferentially, just as does *E. coli* stressed by heat shock, which will lead to a selective production of symbionin (13).

This assumption is compatible with the previous results on

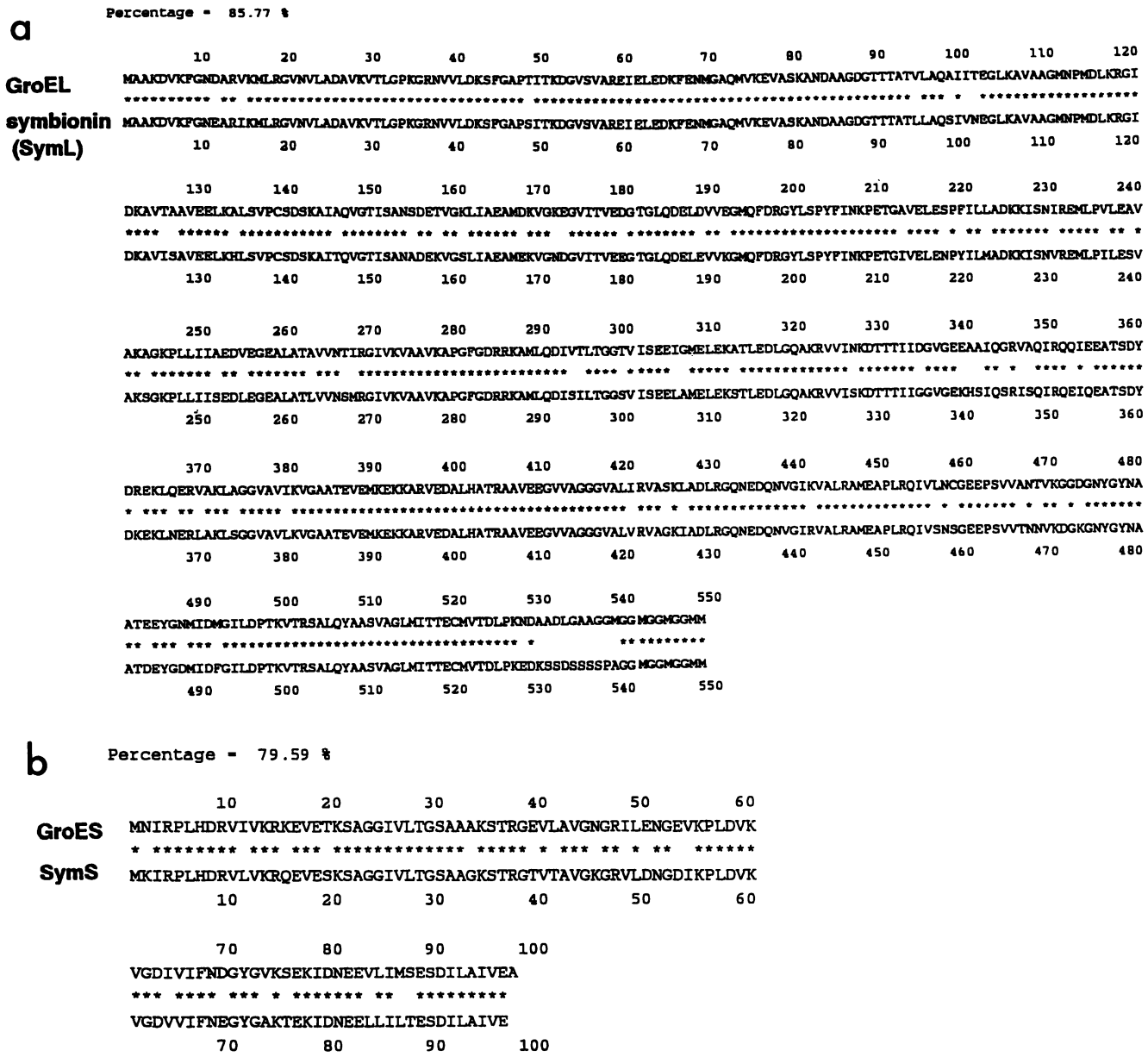


FIG. 2. Comparison between the amino acid sequences of GroEL and symbionin (SymL) and GroES and SymS. Identical amino acid residues are indicated by asterisks. The percent identity is indicated above each alignment.

protein synthesis by the isolated endosymbiont in vitro. Under in vitro conditions, the synthesis of symbionin is markedly lowered and other numerous proteins are produced by the endosymbiont, a phenomenon which is reminiscent of a cell recovered from heat shock (13). In addition, when stressed by heat shock or other stresses such as heavy metals and ethanol, the isolated endosymbiont resumes the selective synthesis of symbionin in large amounts (22). These results suggest that the intracellular environment constrains the endosymbiont to synthesize symbionin preferentially.

As expected from the close similarity of symbionin to GroEL in amino acid sequence (Fig. 2), it was shown that symbionin is functional as an assembly factor in vitro (16) and in vivo in *E. coli*, a heterologous cell (Fig. 3). These findings raise the possibility that in the aphid endosymbiont, symbionin also functions as an assembly factor. Actually, it has been suggested that in the bacteriocyte, many polypeptides synthesized by the cytoplasmic ribosomes are found associated with the endosymbiont (12). Moreover, when the synthesis of symbionin by the endosymbiont is somehow arrested, the endosymbiont structure in the bacteriocyte is destroyed (14, 25). All these, taken together, suggest that symbionin is a molecular chaperone like GroEL and that, just like organellar cpn60s, it is involved in the assembly of polypeptides imported into the endosymbiont from the host cell.

In the complementation experiment shown in Fig. 3, not only *symL* but also *symS* of the endosymbiont was expressed in the *E. coli* mutant cell and complemented the

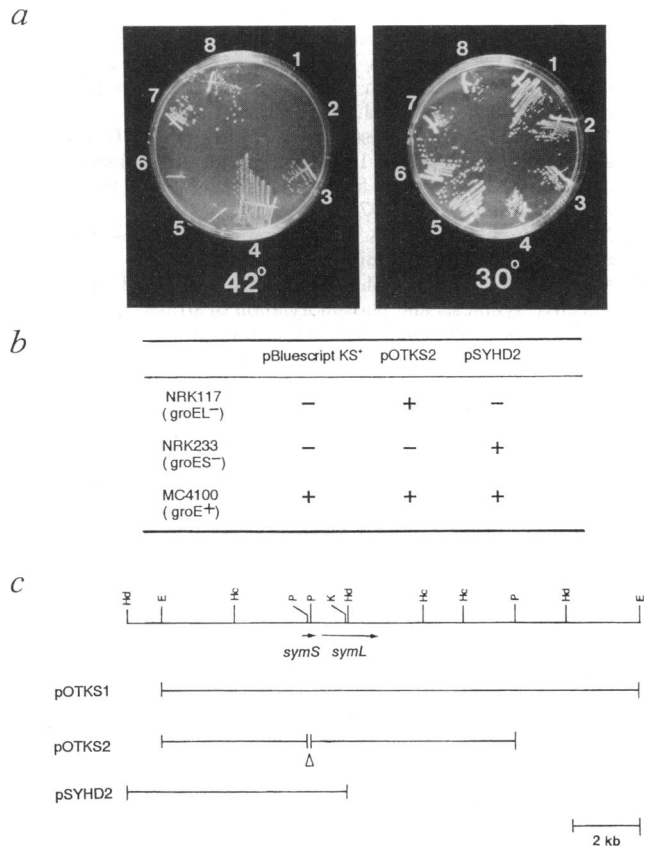


FIG. 3. Restoration of viability at 42°C (a) and phage plaque formation ability (b) of temperature-sensitive *E. coli groEL* and *groES* mutants with *symS* and *symL* expression plasmids. (a) Mutants were transformed with the control and recombinant plasmids shown in panel c. 1 and 2, NRK117 (*groEL*)/pBluescriptKS+; 3 and 4, NRK117/pOTKS2; 5 and 6, NRK233 (*groES*)/pBluescriptKS+; 7 and 8, NRK233/pSYHD2. Plates were incubated at 42°C or 30°C. (b) Transformants were infected with the same number of PFU of λ phage. +, plaques were observed; −, plaques were not observed. (c) Physical map of the *sym* region. The inserted DNA fragments of the recombinant plasmids are indicated below the map. △, deletion obtained by removal of the *Pst*I fragment in the *symS* region. Hd, *Hin*dIII; E, *Eco*RI; Hc, *Hin*cII; P, *Pst*I; K, *Kpn*I.

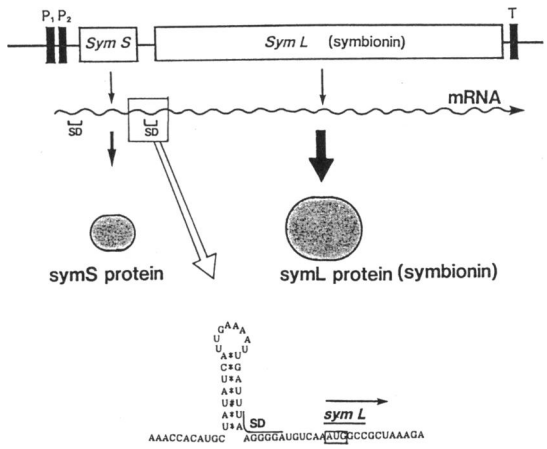


FIG. 4. Model for gene expression of the *sym* operon of the aphid endosymbiont. P1, heat shock promoter; P2, ordinary promoter; T, terminator. A stem-loop structure shown below is assumed to enhance the expression of SymL selectively at the translational level. SD, Shine-Dalgarno sequence.

defect in *groES*. This represents the first example of a functional cpn10 in any heterologous cell. As shown in this experiment, SymS acts like cpn10 in *E. coli*, taking the place of GroES. In addition, symbionin is able to chaperone RuBisCO in vitro, replacing GroEL only in the presence of GroES (16). In this regard, an apparent contradiction is that in the endosymbiont, the synthesis of SymS relative to that of symbionin seems to be very low (16). In *E. coli* (24) and cyanobacteria (33), the level of expression of cpn10 is almost equivalent to that of cpn60.

In mitochondria, no direct evidence is available about the in vivo levels of cpn10, although the presence of a cpn10-like protein has been suggested (20). One possibility is that although cpn10 may be required for some synergistic function with cpn60, cpn10 is not as essential as cpn60 because functions dependent on cpn60 alone are more numerous in organelles. Actually, even in *E. coli*, not all GroEL-dependent processes have been shown to require GroES (1, 19). In mitochondria and chloroplasts, although the cpn60 gene is found in the nuclear genome, the gene encoding cpn10 has not been found in the same operon. In the aphid endosymbiont, the expression of cpn10 (SymS), although encoded in the same operon that encodes cpn60 (symbionin), may be repressed in vivo because of its organellelike environment. Judging from the polarity of the *sym* operon (*symS* arranged upstream of *symL*), the repression of *symS* could be at the posttranscriptional level. In this context, it may be noteworthy that a region preceding the Shine-Dalgarno sequence of *symL* tends to assume a stem-loop structure, which does not seem to be formed either in the Shine-Dalgarno region of *symS* or in the corresponding region of *groE* (Fig. 4). The similar structures are often observed with multicistronic mRNAs of *E. coli* and reported to enhance the translational level of the relevant cistron (21). Further studies of the aphid endosymbiont should provide important insights into the origin of the system in cell organelles, since the endosymbiont is thought to be an organelle at a primitive stage.

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