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Cloning and Characterization of the *groESL* Operon from *Bacillus subtilis*

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The sequence of the 10 N-terminal amino acids of a *Bacillus subtilis* protein that cross-reacts with antibody to *Escherichia coli* GroEL was used to design a set of degenerate oligonucleotide probes. These probes identified a clone which carries almost the entire *groESL* operon from a *B. subtilis* subgenomic library. By chromosomal walking, an additional fragment carrying the 3′ end of *groESL* and its flanking sequence was isolated. Sequence analysis revealed two open reading frames (ORFs) in the cloned DNA. The upstream ORF encodes a 10-kDa protein which has 47% amino acid identity with *E. coli* GroES. The downstream ORF encodes a 58-kDa protein which is 62% identical to *E. coli* GroEL. A 2.1-kb *groESL* mRNA from *B. subtilis* was detected independently by Northern (RNA) blot analyses with a *groES*- and a *groEL*-specific probe. This demonstrated that *groES* and *groEL* are in an operon. The *groESL* promoter was located by using a promoter-probing plasmid, and the apparent transcription start site was mapped by primer extension analysis. The same promoter is utilized under normal and heat shock conditions. This promoter has the same features as a typical σ^A promoter. A strain in which the *groESL* operon was under the control of the sucrose-inducible *sacB* promoter was created. With this strain, it was possible to show that both *groES* and *groEL* are essential genes under both normal and heat shock conditions.

The *groES* and *groEL* genes of *Escherichia coli* are heat shock genes and are organized in an operon (16). They were first identified by mutations affecting the morphogenesis of several bacteriophages (10, 18, 38, 42). The apparent molecular weights of GroES and GroEL are 15,000 and 65,000, respectively. GroEL assembles into a 14-subunit oligomer to form two stacked rings of 7 subunits, each with a central hole (17, 18). The native form of GroES is a single ring of six to eight subunits (6). Recent findings demonstrate that both GroES and GroEL are members of the molecular chaperone family (7). These proteins play an essential role in mediating the correct folding and assembly of many cellular proteins. Besides its interaction with intracellular proteins, *E. coli* GroEL has been shown to form a complex in vitro with several secretory proteins, including pro-OmpA, pre-PhoE, and pre-β-lactamase, and to maintain the export competence of these proteins (3, 21, 25). In vivo, temperature-sensitive *groE* mutations were shown to cause defects in export of pre-β-lactamase (23). Overproduction of GroEL can also facilitate the export of *lacZ* hybrid proteins (31). In fact, both GroES and GroEL are essential for the growth of *E. coli* at all temperatures between 17 and 42°C (9) and are present at high intracellular concentrations (2% of total cellular proteins) under optimal growth conditions (37°C). Furthermore, a family of GroES and GroEL homologs, termed chaperonins, has been found in other bacteria (including eubacteria, archaebacteria, cyanobacteria, and rickettsiae), in the mitochondria of yeast, corn, hamster, rat, mouse, and human; and in the plastids (including chloroplasts, chromoplasts, and etioplasts) of wheat and rape (7). Both the mitochondrial and the plastid chaperonins have been shown to play a vital role in assembling polypeptides that are transported across the membrane.

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Bacillus subtilis, a gram-positive, spore-forming microorganism, is known for its ability to secrete extracellular enzymes directly into the medium in large quantities (32). It serves as an excellent model to study the potential functional roles of GroES and GroEL in protein secretion and sporulation. Furthermore, the regulatory mechanism for controlling the expression of heat shock genes in *B. subtilis* has not been elucidated. It would be interesting to determine whether there is a heat shock sigma factor responsible for transcribing these heat shock genes, as is the case in *E. coli*. To address these questions at the molecular level, cloning and characterization of *groES* and *groEL* from *B. subtilis* would be a prerequisite. Two lines of evidence suggest the existence of a GroEL homolog in *B. subtilis*. An oligomeric form of a 65-kDa protein can be found in *B. subtilis* under physiological conditions (5). This complex has a morphology (i.e., a ringlike structure with seven 65-kDa subunits per ring), dimensions, and a sedimentation coefficient similar to those for the *E. coli* GroEL oligomer. It can be immunoprecipitated by using antiserum against *E. coli* GroEL. This complex was found to copurify with a viral protein (P10) that is involved in the early steps of phage head assembly when the cells were infected with phage φ29. Furthermore, under heat shock conditions, a 65-kDa protein from *B. subtilis* shows cross-reaction with anti-*E. coli* GroEL antiserum (1). In this report, we describe the cloning and characterization of the *groESL* operon from *B. subtilis*. The promoter utilized under normal and heat shock conditions was determined, and the essential role of *groES* and *groEL* for cell growth was established.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The *B. subtilis* strains used in this study were 168 (*trpC2*) for chromosomal DNA preparation, DB104 (*his nprR2 nprE18*

aprΔ3) (20) for routine transformations, and WB30 [*his nprR2 nprE18 aprΔ3 sacU200*(Hy)] (49) for the expression studies. *E. coli* DH5α [(ϕ80d*lacZΔM15*) *endA1 recA1 hsdR17* (r^- m^-) *supE44 thi-1* λ^- *gyrA relA1* F^- Δ(*lacZYA-argF*)*U169*] was used as a host for the construction of subgenomic libraries. Plasmid pUB18 (50), a pUB110 derivative carrying a polylinker from pUC18, was used for routine subcloning in *B. subtilis*. *E. coli* Bluescribe plasmid (pBS; from Stratagene) was used for the construction of subgenomic libraries and routine subcloning. Plasmid pT7-6 (41) was used for the expression of the *E. coli groESL* operon. Plasmid DNA was transformed by the standard competent-cell methods for *B. subtilis* and *E. coli* as described previously (51). *B. subtilis* was cultured in either superrich medium (13) or L broth. *E. coli* was cultured by standard microbiological methods. For heat shock studies, cells were cultured in the superrich medium at 37°C with the cell density up to 0.3 to 0.5 optical density unit (at 600 nm). Half of the sample was transferred to 50°C, while the remaining half was kept at 37°C.

DNA manipulations. The preparation of bacterial (*E. coli* and *B. subtilis*) chromosomal and plasmid DNAs, Southern and colony hybridization, and DNA sequencing were performed as described previously (52). Primer extension was performed as described by Moran (29), with the reverse transcription reaction performed at 45°C. A random primer labeling kit, restriction enzymes, and DNA modification enzymes were purchased from Bethesda Research Laboratory, Pharmacia, and Boehringer Mannheim and used according to the recommendations of the manufacturers. Two sequence analysis packages were used in this study. They were Microgenie (version 6.0) from Beckman and PC gene (version 6.6) from Intelligenetics.

Construction of pT7-GROESL. To obtain a sufficient quantity of *E. coli* GroEL for antibody preparation, the pT7 expression system was used (41). The *E. coli groE* operon was generated through the polymerase chain reaction (PCR) with the template DNA from JM101. Two primers were designed on the basis of the published sequence of *E. coli groESL* operon (16). The forward primer (5′ AAGCTTCTC TGGTCACCAGCCGGGA 3′) was 25 nucleotides long and covered the 5′ end of the operon starting 66 nucleotides upstream of the putative *groES* ribosome-binding site (16). A *Hin*dIII site was introduced at the 5′ end of this sequence. The backward primer (5′ GCTTGAATTCTGCGAGGTG CAGGGCAATTAC 3′) was 31 nucleotides long and covered the translation termination codon of *groEL*. An *Eco*RI restriction site was introduced at the 5′ end of this primer. A GeneAmp kit from Perkin-Elmer Cetus was used for the amplification (33) under the following conditions: 93°C for 1 min, 54°C for 1 min, and 72°C for 2.5 min (30 cycles) with a programmable Dri-block (PHC-1) from Techne. The amplified 2.1-kb fragment was digested with *Hin*dIII and *Eco*RI and ligated to *Hin*dIII-*Eco*RI-double-digested plasmid pT7-6 to generate pT7-GROESL.

Production, purification, and preparation of anti-GroEL antibody. Overproduction of both GroES and GroEL was observed under the induction condition after the transformation of pT7-GROESL into *E. coli* K38 carrying pGP1-2 (41). The bacterial proteins were separated on a sodium dodecyl sulfate (SDS)–12% polyacrylamide gel (24). The 65-kDa protein band corresponding to GroEL was excised and electroeluted (52). The eluted sample was then applied to a 12% native polyacrylamide gel, and the major protein band was dissected and eluted. The purified GroEL with a purity over 95% was mixed with complete adjuvant and injected into mice to prepare antibody.

N-terminal sequence determination of the *B. subtilis* GroEL homolog. *B. subtilis* 168 was cultured under the heat shock condition for 2 h, and the total cellular proteins were resolved on a preparative SDS–12% polyacrylamide gel with the conditions specified by Matsudaira (26). After the gel was stained with Coomassie blue for 10 min, a major protein band with a size of 65 kDa was observed. This protein band was sliced into three pieces (upper, middle, and lower), and protein was electroeluted from the gel (52). The purity of the eluted protein was determined by SDS-polyacrylamide gel electrophoresis. A single protein band was detected from one of the samples. The identity of this protein as the GroEL homolog was confirmed by Western blot (immunoblot) analysis with anti-*E. coli* GroEL antiserum. This protein was electroblotted to the Immobilon membrane (26), and the N-terminal sequence of this protein was determined by a gas-phase sequencer at the microchemistry center at the University of Victoria. The sequence for the first 30 amino acids was determined.

Design of *B. subtilis* GroEL-specific oligonucleotide probes. Based on the sequence of the first 10 amino acids determined from the *B. subtilis* GroEL, a set of degenerate oligonucleotide probes with the following sequence was synthesized at the regional DNA synthesis laboratory at the University of Calgary: 5′ ATGGCIAA(AG)GA(AG)AT(CT)AA(AG)TT(CT) (TA)(CG)IGA(AG)GA(AG)GC 3′. These probes were 32 nucleotides long.

Northern blot hybridization. RNA was isolated from *B. subtilis* under normal and heat shock conditions (5, 10, 20, and 35 min after heat shock) by the method of Gilman et al. (11). Two sets of RNA samples were loaded to the formaldehyde gel, and a Northern (RNA) blot was performed as described by Moran (29). After blotting, the filter was sliced into two. One was hybridized with the 345-bp *groES* probe (nucleotides 293 to 638) which was generated through PCR with P2 and P5 as primers and *B. subtilis* 168 chromosomal DNA as the template. The other was hybridized with the 1.16-kb *Xba*I-*Pst*I *groEL* probe (Fig. 1). Both probes were labeled by using the random primer labeling kit (Bethesda Research Laboratories) by following the recommendations of the manufacturer. Excess probes were used for hybridization in each case. After being washed, the two filters were reassembled and analyzed by autoradiography. To quantitate the mRNA level of *groESL*, the radioactive band from each lane was excised from the filter and the radioactivity was determined by liquid scintillation counting.

Construction of pCAT and pCAT-PG for promoter mapping and the CAT assay. *B. subtilis* plasmid pCAT is a pUB18 derivative carrying a promoterless chloramphenicol acetyltransferase gene (*cat*) from pC194 (19). Since no unique restriction enzyme sites are located between the *cat* promoter and the ribosome-binding site, a set of PCR primers was synthesized to generate the promoterless *cat* cassette with a *Pst*I site at the 5′ end and a *Hin*dIII site at the 3′ end. The sequence for the forward primer is as follows: 5′ CGGGATCCTGCAGTAGCAGACAAGTAAGCCTCC 3′. The sequence from nucleotides 15 to 33 in the primer corresponds to nucleotides 1204 to 1221 in pC194. The backward primer has the following sequence: 5′ CGTCTA GAAGCTTCTTCAACTAACGGGGC 3′. Nucleotides 12 to 29 in the primer correspond to the 3′ end of *cat* in pC194 (nucleotides 1966 to 1984). The amplified cassette was digested with *Pst*I and *Hin*dIII and ligated to *Pst*I-*Hin*dIII-

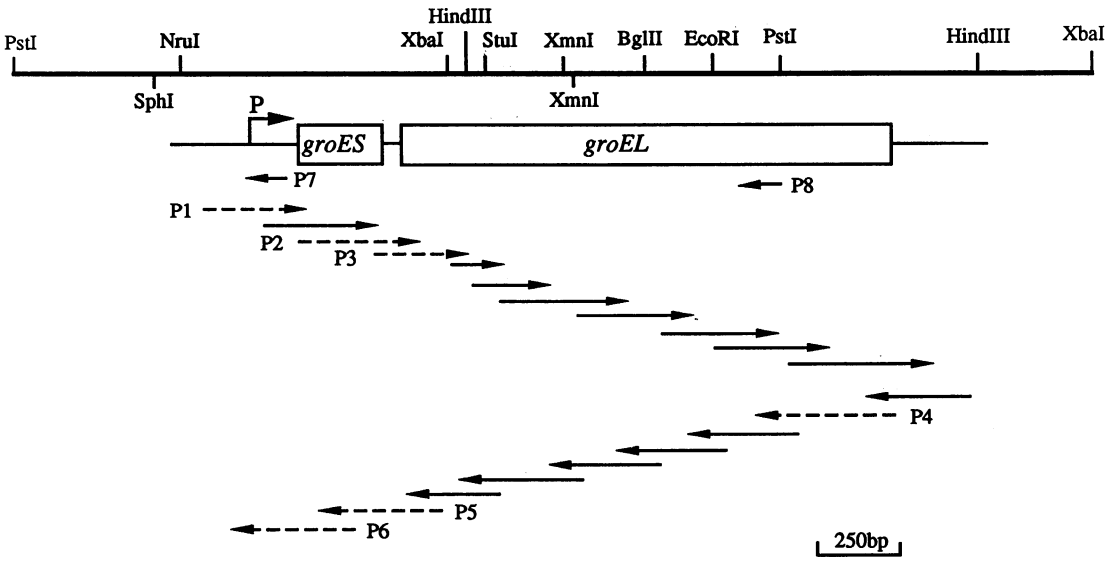

FIG. 1. Restriction map and sequencing strategy of the *B. subtilis groESL* operon. The 3.2-kb *Pst*I fragment was from pGROESL. The 1.7-kb *Eco*RI-*Xba*I fragment (on the right) was from pGD. Restriction sites used for subcloning are indicated. Dashed lines indicate the extent of the sequence determined with synthetic primers. The open reading frames for *groES* and *groEL* are boxed. P represents the promoter region. Primers used for sequencing are numbered from P1 to P6. P7 is one of the primers used in the amplification of the promoter region of *groESL*, and P8 is the hybridization primer to isolate the 3′ end of the *groESL* operon.

digested pUB18 to generate pCAT. To introduce the promoter region from the *B. subtilis groESL* operon into this plasmid, a promoter-containing (*Nru*I-*Bam*HI) fragment was amplified by a pair of sequencing primers, P1 and P7: 5′ CGAGATCTGGATCCAATTCTTATAATAAAGAATCTC 3′ (nucleotides 266 to 288), as shown in Fig. 1. The amplified fragment was digested by *Rsa*I and *Bam*HI, and the 110-bp *Rsa*I-*Bam*HI fragment was ligated to the *Sma*I-*Bam*HI-digested pCAT to generate pCAT-PG. The chloramphenicol acetyltransferase (CAT) activity was determined by the method of Shaw (34). One unit of CAT activity was defined as the amount of enzyme required to acetylate 1 μmol of chloramphenicol per min at 37°C.

Construction of pBM4 and pSACB-GROESL. Plasmid pBM4 was designed to replace the chromosomal *groESL* with a modified *groESL* operon. The expression of this modified *groESL* operon was controlled by the inducible promoter from *sacB*, a structural gene for levansucrase. This plasmid has four key components, including a *Sph*I fragment carrying the sequence upstream of the *groESL* operon, a *cat* gene as a selection marker, a *sacB* promoter, and the promoterless *groESL* operon (see Fig. 6A). A 1.2-kb *cat* cassette originally derived from pC194 was obtained from pAZ111 (30) by *Pst*I and *Xba*I digestion. This cassette was ligated with *Pst*I-*Xba*I-digested pBS to generate pBM1. A 1.2-kb *Sph*I fragment carrying the sequence upstream of the *groESL* operon was obtained through a *Sph*I digestion of pUG, which was a pBS derivative carrying the *Pst*I-*Nru*I fragment (the left arm of the cloned 3.2-kb *Pst*I fragment, as shown in Fig. 1). This fragment was inserted into pBM1 to generate pBM2. The correct orientation of the inserted *Sph*I fragment in pBM2 was confirmed by sequencing. A 550-bp *Bam*HI-*Sna*BI fragment carrying the sucrose-inducible *sacB* promoter region was obtained by PCR amplification of *B. subtilis* 168 DNA with a pair of primers. The sequence of the forward primer was as follows: 5′ CGG TACCTCTAGAGGATCCCATCACATATACCTGCCG 3′. Nucleotides 18 to 37 in this sequence correspond to nucleotides 13 to 32 of the published *sacB* sequence (37). This sequence is upstream of the *sacB* promoter. The backward primer had the following sequence: 5′ CCATATGTAAT GTCCTCCTTTTTTACGTACTGTG 3′. Nucleotides 15 to 34 correspond to the sequence of the ribosome-binding site region of *sacB* (nucleotides 435 to 454) on the complementary strand. The amplified fragment was digested by *Bam*HI and *Sna*BI and ligated with the *Bam*HI-*Sma*I-digested pBM2 to generate pBM3. The last step was the insertion of a 2.1-kb *Kpn*I-*Sac*I fragment carrying the promoterless *groESL* into pBM3 to generate pBM4. This *groESL* fragment was also generated by PCR with a pair of primers (P2, 5′ GGGGTAC CAGCACTCTTTAGTGCTGAGTG 3′, primer sequence 9 to 29 corresponds to nucleotides 293 to 314 in Fig. 2; P4, 5′ GGGGTACCGAGCTCATATGACCGCTAATATGG 3′ with the sequence corresponds to nucleotides 2454 to 2438).

Plasmid pSACB-GROESL was constructed by ligating the *Bam*HI-*Sac*I cassette from pBM4 into pUB18. This plasmid allows the detection of a high-level production of GroES and GroEL in *B. subtilis* by adding sucrose to the culture medium.

Replacement of chromosomal *groESL* with the modified *groESL* from pBM4. Plasmid pBM4 was linearized by *Sac*I and transformed into DB104 and WB30. Cells were selected on a tryptose blood agar base plate containing 5 μg of chloramphenicol per ml and 2% sucrose. The exchange of the chromosomal *groESL* with the modified *groESL* from pBM4 in the mutant strains was confirmed by PCR analysis (with P1 and P6 as the amplification primers, as shown in Fig. 6; the sequence of P6 corresponds to nucleotides 369 to 387, as shown in Fig. 2) and Southern hybridization with the 2.1-kb *Kpn*I-*Sac*I *groESL* fragment and the *cat* cassette as hybridization probes.

Nucleotide sequence accession number. The nucleotide sequence reported in this article has been assigned GenBank accession number M81132.

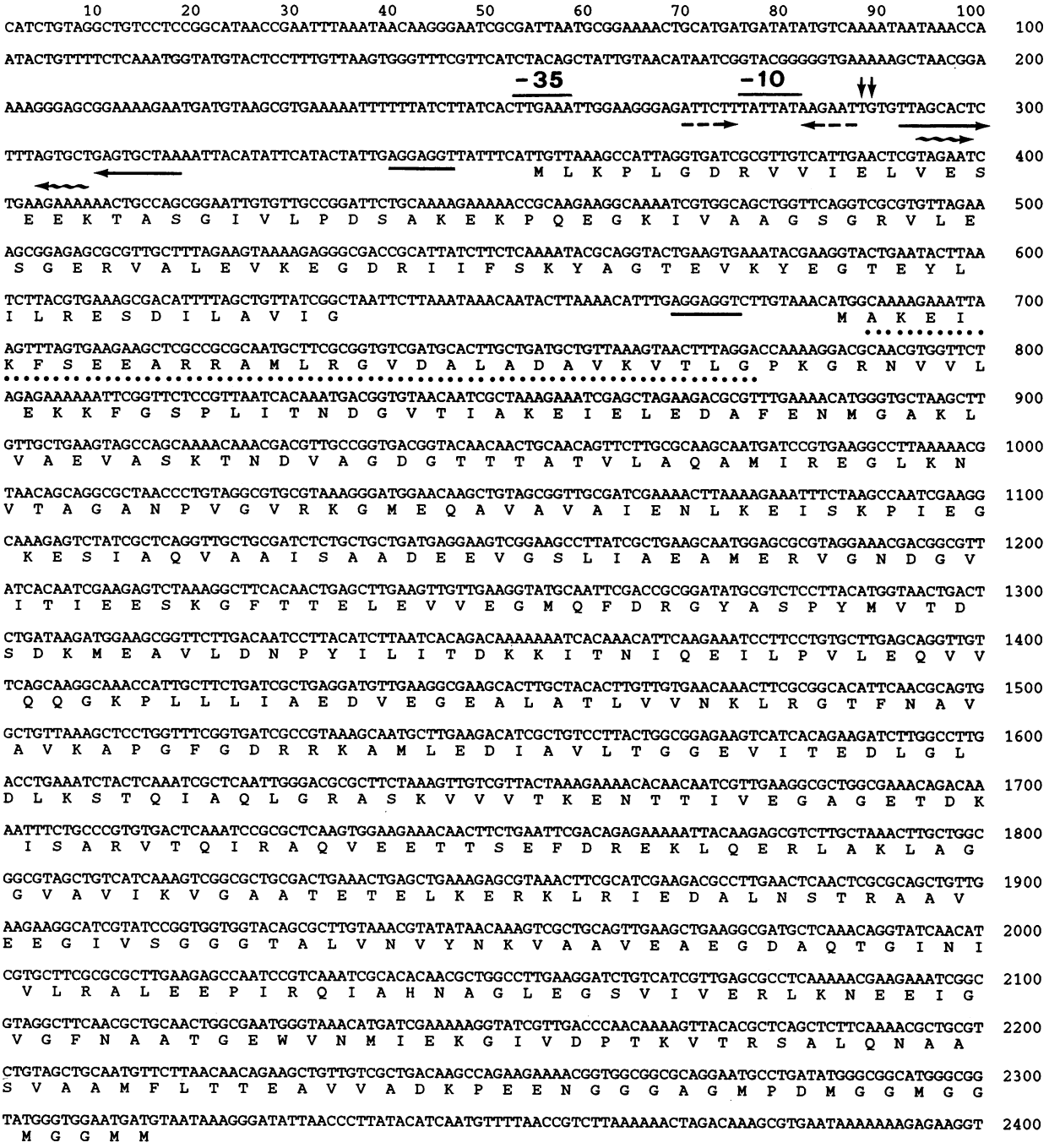

FIG. 2. Nucleotide sequence of *B. subtilis groESL*. The open reading frames of *groES* and *groEL* extend from bases 354 to 638 and 685 to 2322, respectively. The deduced amino acid sequence for each open reading frame is shown below the DNA sequence. The putative ribosome-binding sites are underlined. The amino acid sequence marked by a dotted line represents the sequence determined from direct sequencing of GroEL. The −10 and −35 regions for the *groESL* operon are marked. The transcription start sites, determined by primer extension analysis, are indicated with vertical arrows. Inverted repeats 1, 2, and 3 are underlined by broken, solid, and wavy arrows, respectively.

RESULTS

Cloning of *groESL*. The first step towards the cloning of the *B. subtilis groEL* was the identification and purification of the 65-kDa heat shock protein that cross-reacted with the anti-*E. coli* GroEL antibody. The sequence of the first 30 amino acids from the N terminus of this protein was determined (Fig. 2). Based on the sequence of the first 10 amino acids of this protein, a set of oligonucleotide probes was designed and applied to hybridize with *B. subtilis* chromosomal DNA digested with *Eco*RI, *Pst*I, or *Hin*dIII. A 4.0-

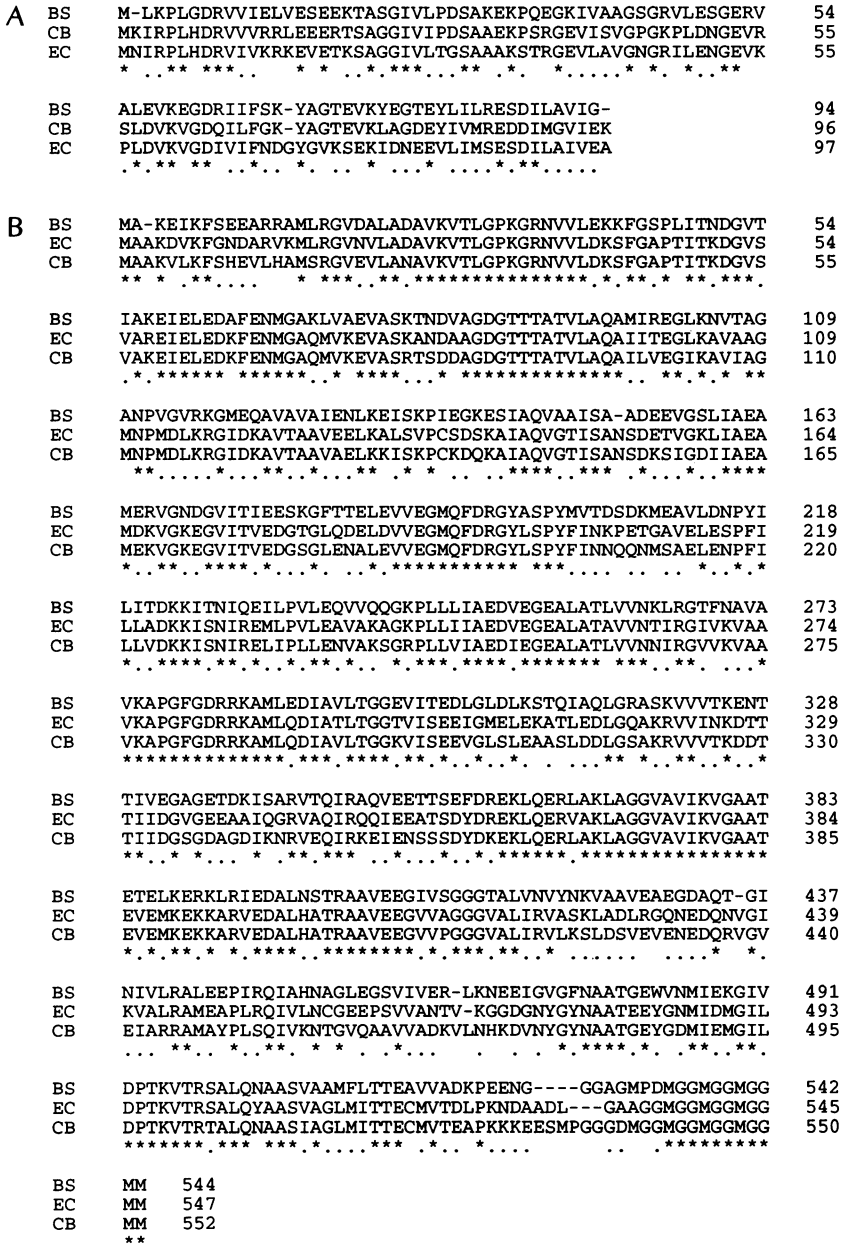

FIG. 3. Sequence alignment of *B. subtilis* GroES (A) and GroEL (B) with their homologs. Identical residues are indicated by asterisks. Conserved residues are indicated by dots. BS, *B. subtilis*; EC, *E. coli*; CB, *C. burnetii*.

kb *Eco*RI fragment, a 3.2-kb *Pst*I fragment, and a 4.3-kb *Hin*dIII fragment hybridized specifically with the probes (data not shown). The *B. subtilis* chromosomal DNA was then digested by *Pst*I and size fractionated. Fragments with sizes of around 3.2 kb were ligated to the Bluescribe plasmid (pBS) to generate a subgenomic library. One positive clone (pGROESL) was identified through colony hybridization. This clone has four *Pst*I inserts. Two of the inserts could be separated from the *groESL* fragment by size fractionation on a 1% agarose gel. The remaining one could be separated from the *groESL* fragment after a *Bam*HI digestion. This digestion did not affect the *groESL* fragment but did split the other into two smaller fragments. However, the purified, intact *groESL* fragment could not be cloned in either pBS or pUB18. All of the resulting clones were found to have significant deletions within this fragment. Subcloning different portions of the *groESL* fragment into pBS was possible except for the sequence between nucleotides 192 to 324, as shown in Fig. 2. Sequence analysis indicated that the 3′ end of the *groESL* operon was not included in the cloned 3.2-kb *Pst*I fragment. To clone the 3′ end of the *groESL* operon, an oligonucleotide with the sequence corresponding to the 3′ end of the 3.2-kb *Pst*I fragment was synthesized (nucleotides 1916 to 1937, P8). This primer hybridized specifically to a 1.7-kb *Eco*RI-*Xba*I fragment from the *B. subtilis* genome. A subgenomic library with the enrichment of the 1.7-kb *Eco*RI-

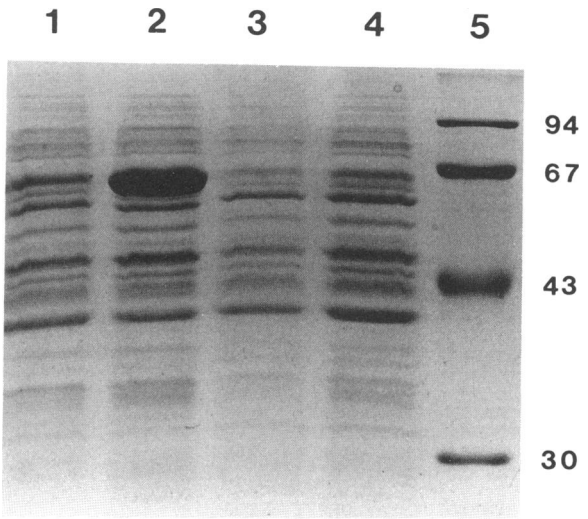

FIG. 4. Overproduction of *B. subtilis* GroEL. *B. subtilis* strains were cultivated in superrich medium with or without 2% sucrose, and the total intracellular proteins were analyzed on an SDS–12% polyacrylamide gel. Lane 1, noninduced WB30(pSACB-GROESL); lane 2, sucrose-induced WB30(pSACB-GROESL); lane 3, noninduced WB30(pUB18); lane 4, sucrose-induced WB30(pUB18); lane 5, molecular size markers (indicated on the right in kilodaltons).

*Xba*I fragments was constructed with the pBS plasmid as the cloning vector. Three positive clones were identified by colony hybridization. One of the recombinant plasmids (pGD) was used for further characterization.

Characterization of the *groESL* operon. Figure 1 shows the restriction map of the *groESL* operon. The DNA sequence of the operon was determined on both strands. The sequence of the region that could not be subcloned was determined by two methods: (i) sequencing pGROESL with two synthetic oligonucleotides (P1 and P6) and (ii) direct sequencing (with P6 as the sequencing primer) of the amplified *groESL* fragment (amplified by P1 and P5; Fig. 1) with *B. subtilis* 168 chromosomal DNA as the template (22). An identical sequence in this region was obtained. This result confirmed that there is no sequence rearrangement within this unclonable region from the 3.2-kb *groESL* fragment. The nucleotide sequence of the *groESL* operon and its flanking regions is shown in Fig. 2. Two open reading frames were found. One started with a TTG codon at nucleotide 354 and ended with a TAA codon at nucleotide 638 (94 amino acids; M_r = 10,170). The TTG codon is preceded by a putative ribosome-binding site (AGGAGGT) with the calculated $\Delta G'$ of −18 kcal (ca. −73.8 kJ)/mol (43). This protein demonstrated 47% amino acid identity with *E. coli* GroES (16) and 58% identity with the 10-kDa antigen protein of *Mycobacterium tuberculosis* (2). The second open reading frame started with an ATG codon at nucleotide 685 and terminated at nucleotide 2317 with two consecutive TAA codons (544 amino acids; M_r = 57,440). A putative ribosome-binding site (AGGAGGT) was located 9 bp upstream of the ATG codon. This protein showed 61.4% amino acid identity with *E. coli* GroEL (16), 63.5% with the 65-kDa antigen of *M. tuberculosis* (35), 61% with the *Synechoccus* GroEL homolog (45), and 60% with the heat shock protein B of *Coxiella burnetii* (44). The sequence alignment of *B. subtilis* GroES and GroEL with other chaperonins is shown in Fig. 3. The deduced N-terminal sequence of GroEL from positions 2 to 31 exactly matches that determined by direct sequencing of GroEL (Fig. 2). This result indicated that the first methionine residue of GroEL was removed in vivo by methionine aminopeptidase. Miller et al. (28) recently reported the N-terminal sequences of two internal peptides from *B. subtilis* GroEL. These sequences showed good agreement with our deduced GroEL sequence (residues 191 to 205 and 327 to 345). Overproduction of a 65-kDa GroEL from WB30 (pSACB-GROESL) was observed under induction conditions (Fig. 4). Similar to the case observed in *E. coli*, the apparent molecular weight of the *B. subtilis* GroEL (M_r = 65,000) is larger than that calculated from the sequence data. Production of GroES from WB30(pSACB-GROESL) could also be observed when a larger quantity of protein sample was loaded to the gel (data not shown).

***groES* and *groEL* are cotranscribed.** To determine whether *groES* and *groEL* are organized in an operon, as is the case in *E. coli*, two hybridization probes were used in the Northern blot analyses with the *B. subtilis* mRNA isolated under normal (37°C) and heat shock (50°C) conditions. One was *groES* specific, and the other was *groEL* specific. Each probe hybridized to a single mRNA 2.1 kb in length (Fig. 5A). The use of a 2.1-kb *Kpn*I-*Sac*I probe carrying both *groES* and *groEL* gave the same result (data not shown). This demonstrated that *B. subtilis groES* and *groEL* are arranged in a bicistronic operon. The kinetics of accumulation of *groESL* mRNA were determined by the Northern blot analysis with the 345-bp *groES* probe. The *groESL* mRNA increased rapidly within 5 min after heat shock and reached a maximum level (an 18-fold increase above the background level) within 10 min (Fig. 5B). It was then decreased gradually. Comparable kinetics were obtained with the *groEL*-specific probe (data not shown).

Mapping the transcription start site for the *groESL* operon under heat shock and non-heat-shock conditions. A typical σ^A (σ^{43}) promoter sequence could be found upstream of *groES* (Fig. 2). The spacer region is 17 bp long between the putative −35 (TTGAAA at nucleotides 253 to 258) and −10 (TAT TAT at nucleotides 276 to 281) regions. Three sets of inverted repeats (IR1 to IR3) were identified around the promoter region (Fig. 2). To test whether this sequence can function as a promoter, the putative promoter-containing fragment (nucleotides 175 to 288, 103 bp) was inserted into a promoter-probing plasmid, pCAT, which carries the promoterless *cat* from pC194. The resulting plasmid was named pCAT-PG. Specific CAT activity (240 mU/mg of total cellular protein) was observed with the lysate of *B. subtilis* DB104(pCAT-PG), while the control, DB104(pCAT), showed no detectable CAT activity. Attempts to clone the PCR-amplified promoter fragment carrying the inverted repeats around the regulatory region (nucleotides 175 to 375, 200 bp) into pBS, pUB18, and pCAT failed. Deletion within the cloned fragment was observed. To map precisely the transcription start site of the *groESL* operon, a 19-nucleotide primer, P6 (with the sequence complementary to nucleotides 369 to 387), was synthesized for primer extension analysis with RNA isolated from *B. subtilis* 168 under heat shock (35 min at 50°C) and non-heat-shock conditions. Two transcription start sites which differed from each other by 1 nucleotide were observed, and the same two transcription start sites were utilized under both conditions (Fig. 5C). This result suggested that transcripts began with either a T (nucleotide 288) or a G (nucleotide 289) and that the pre-

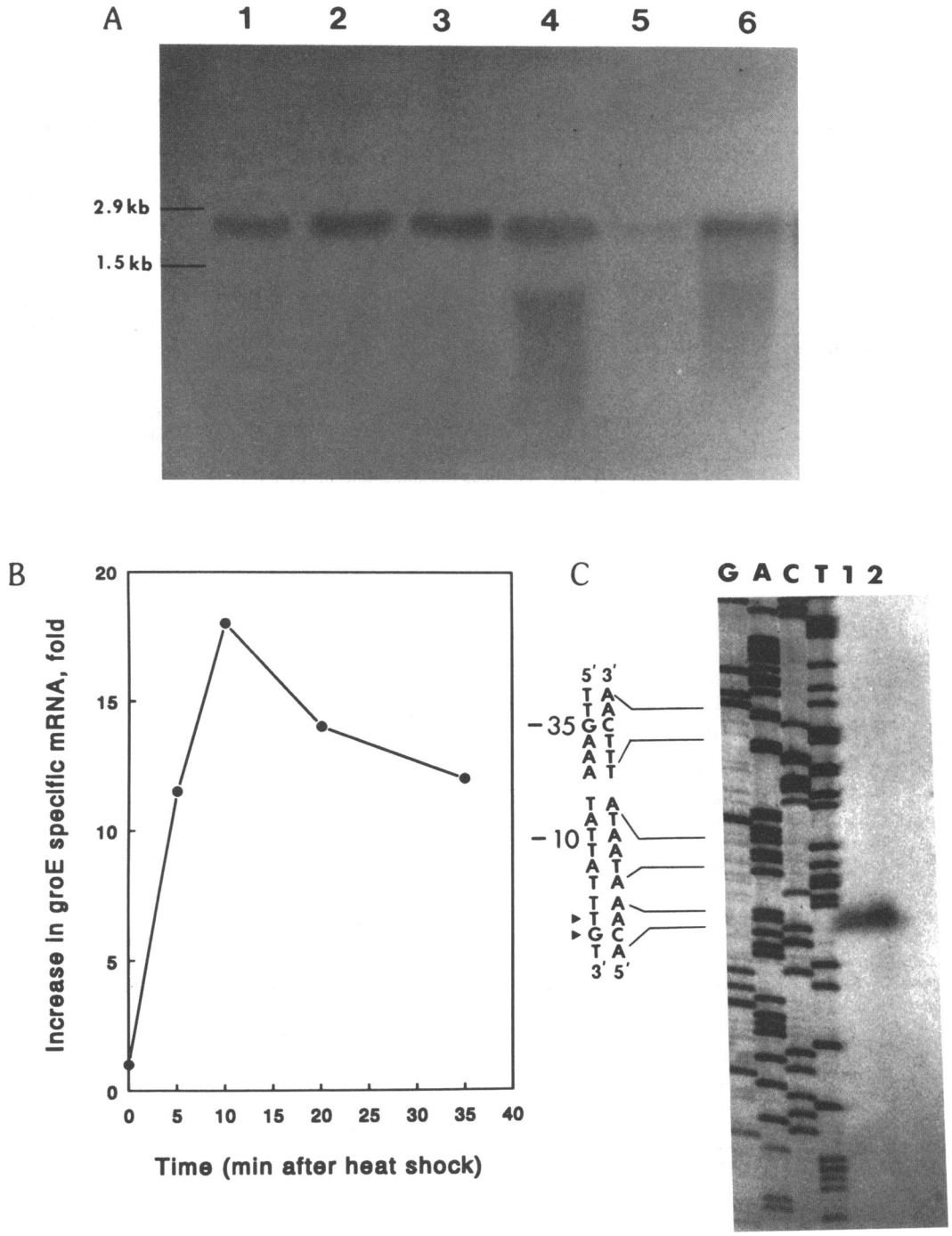

FIG. 5. Analysis of *groESL* transcript. (A) Northern blot analysis of *groESL* transcript from *B. subtilis* DB104. Lanes 1 to 4, hybridization of RNA isolated 35, 20, 10, and 5 min, respectively, after heat shock (50°C) with the 345-bp *groES* probe (nucleotides 293 to 638). Lane 5, RNA from DB104 cultivated at 37°C with the cell density up to 0.3 optical density unit (at 600 nm). The 345-bp *groES* fragment was used as the hybridization probe. The amount of RNA in lanes 4 and 5 was three times more than that in lanes 1 to 3. Lane 6, hybridization of RNA isolated 35 min after heat shock with the 1.16-kb *Xba*I-*Pst*I *groEL* probe. 23S and 16S rRNA are used as sizing markers. (B) Kinetics of *groE* mRNA accumulation after heat shock. Data were obtained by counting each radioactive band in panel A and normalized with the amount of RNA loaded in each lane. (C) Transcription start site determination by primer extension analysis. Lanes 1 and 2, primer-extended products observed with RNA templates prepared from cells under non-heat-shock (37°C) and heat shock (50°C for 35 min) conditions. Relative to amount of the heat shock RNA, eight times more non-heat-shock RNA was used as a template. The four lanes on the left are the sequencing ladders generated from pGROESL with P6 as the sequencing primer. P6 is also the primer used in this primer extension experiment. The −35 and −10 regions of *groESL* are marked, and the two arrowheads mark the nucleotide sequences at the transcription start sites.

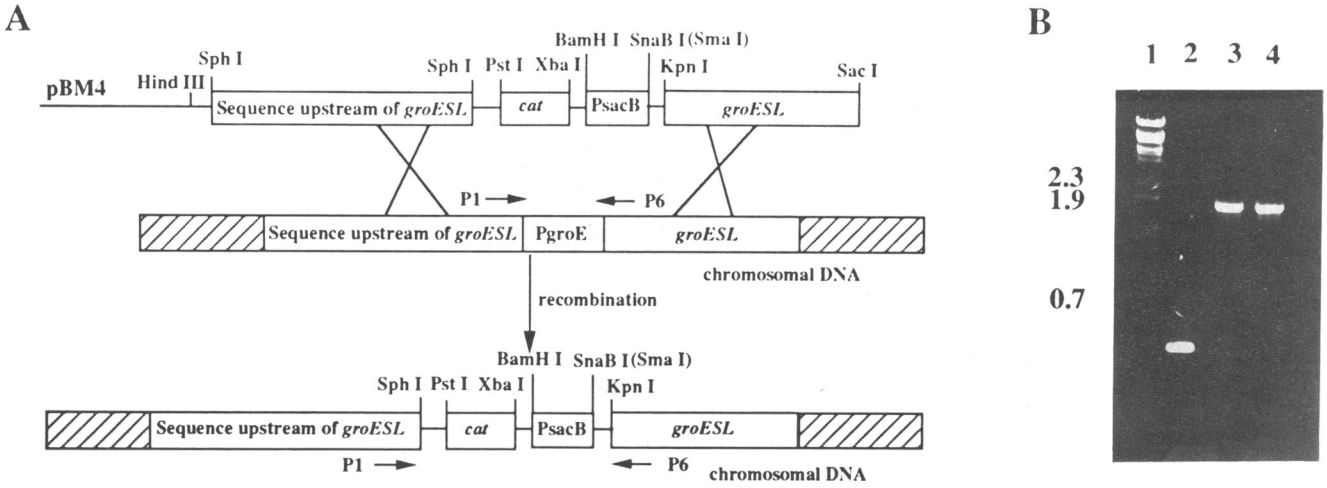

FIG. 6. Construction of *B. subtilis* MB2 and WB30C. (A) Replacement of the chromosomal *groESL* operon by *Sac*I-digested pBM4; (B) confirmation for the construction of *B. subtilis* MB2 and WB30C by PCR amplification. Lane 1, *Bst*EII-digested lambda DNA as molecular markers; lanes 2 to 4, amplification of chromosomal *groESL* from *B. subtilis* DB104, MB2, and WB30C, respectively. P1 and P6 as shown in panel A were the primers used for the amplification analysis. Sizes are indicated on the left (in kilobases).

dicted σ^A type promoter was indeed functional under these conditions.

Construction of *B. subtilis* MB2 and WB30C. To determine whether *groES* and *groEL* are essential genes in *B. subtilis*, the chromosomal *groESL* operon in DB104 and WB30 [a DB104 derivative carrying a *sacU*(Hy) mutation which stimulates transcription from the *sacB* promoter] was replaced by a modified *groESL* operon with its expression under the control of the sucrose-inducible *sacB* promoter (Fig. 6). The resulting mutant strains were named MB2 (DB104 with a modified *groESL*) and WB30C (WB30 with a modified *groESL*), respectively. The ability of these strains to grow at different temperatures in the presence and absence of 2% sucrose was then monitored. To confirm that *groESL* had been replaced in these strains, chromosomal DNA from MB2 and WB30C was prepared and amplified by a pair of primers (P1 and P6; Fig. 6A). Figure 6B shows that a shift in size for the amplified fragment from DB104 (300 bp) in comparison with those from MB2 (1.8 kb: 1.2 kb from *cat* and 0.6 kb from the *sacB* promoter) and WB30C (1.8 kb) was observed. Further confirmation was demonstrated by Southern hybridization with the labeled 2.1-kb *Kpn*I-*Sac*I *groESL* fragment (carrying both *groES* and *groEL*) and the *cat* cassette (data not shown).

***groES* and *groEL* are essential genes for cell growth under normal and heat shock conditions.** Figure 7A compares the growth of DB104, MB2, WB30, and WB30C at 37°C in superrich medium with or without sucrose. Strains (DB104 and WB30) carrying the wild-type *groESL* grew well and showed identical growth patterns in superrich medium with or without sucrose. Although MB2 did not grow as well as DB104, it was at least able to grow in the presence of 2% sucrose. No significant cell growth could be observed in the absence of sucrose. This suggested that the expression of *groESL* was required for normal growth. Since the *sacU*(Hy) mutation is known to enhance the transcription from the *sacB* promoter (49), a higher level of GroES and GroEL would be expected in WB30C. This might in turn allow WB30C to grow better than MB2. Indeed, that was the case. In the presence of sucrose, WB30C grew as well as did DB104. A low level of cell growth for WB30C was observed even in the absence of sucrose. Figure 7B compares the cell growth at 50°C. DB104 (and WB30) grew well under this condition, independent of whether sucrose was present. No growth could be observed for MB2 in either the presence or absence of sucrose. WB30C was able to grow in the presence of sucrose, although its highest cell density was only 60% of that observed for DB104. Poor growth of WB30C was the result in the absence of sucrose. The difference in growth patterns observed for WB30C and MB2 was expected to be due to the higher level of GroES and GroEL production in WB30C. To determine whether this assumption was valid, Western blot analysis was applied to quantitate the level of GroEL from the samples of DB104, WB30, MB2, and WB30C prepared under normal and heat shock conditions (Fig. 7C and D). A direct relationship between the ability of the cells to grow and the level of GroEL could be observed. These data clearly demonstrated that the expression of functional GroEL (and GroES) in sufficient quantity is required to support cell growth under normal and heat shock conditions. An extra protein band with the size of 39 kDa was observed in the samples of DB104 and MB2 under the non-heat-shock condition. The identity of this band is unknown, but it may be a degraded form of GroEL.

DISCUSSION

In *B. subtilis* 168, induction of heat shock proteins can be observed 3 min after heat shock and a decline of non-heat-shock proteins is observed within 6 min (1). There are at least 16 heat shock proteins, including homologs of DnaK, GroEL, LonA, enolase, and glyceraldehyde-3-phosphate dehydrogenase (1, 28). Their sizes range from 4.5 to 138 kDa. Similar to that in *E. coli*, the heat shock response in *B. subtilis* is a transient response. The *B. subtilis groESL* mRNA accumulated rapidly and reached the maximum level within 10 min after heat shock (Fig. 5B). The mRNA level was then decreased with time. This kinetic profile correlated very well with that of a 66-kDa heat shock protein (presum-

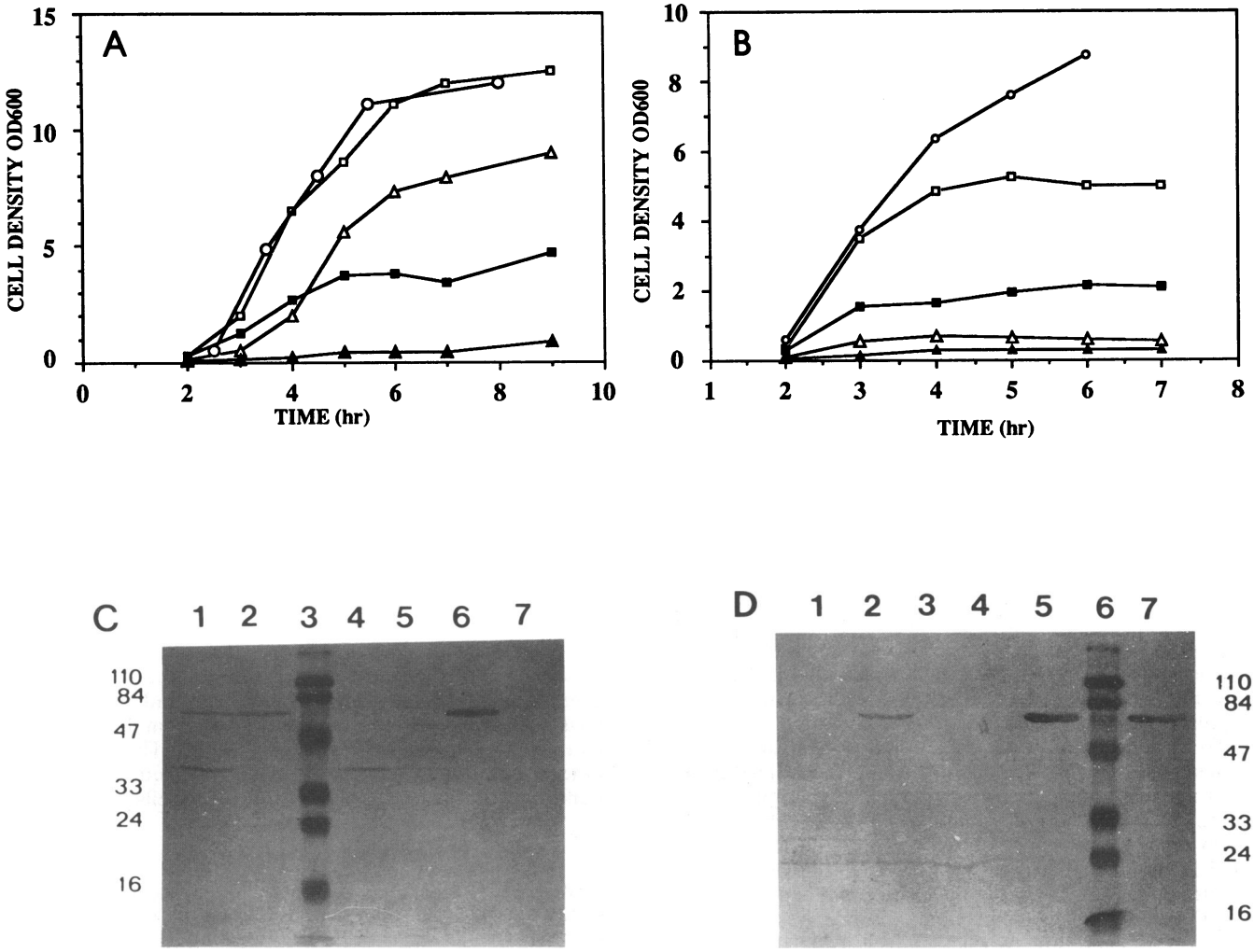

FIG. 7. *groES* and *groEL* are essential genes. (A and B) Growth of DB104, MB2, and WB30C in superrich medium at 37°C (A) or 50°C (B) in the presence or absence of sucrose. Circle, DB104 (or WB30) with or without sucrose; closed triangle, MB2 without sucrose; open triangle, MB2 with sucrose; closed square, WB30C without sucrose; open square, WB30C with sucrose. OD600, optical density units (at 600 nm). (C) Western blot analysis of GroEL production in DB104, MB2, and WB30C cultured in superrich medium at 37°C. Lane 1, DB104 without sucrose; lane 2, WB30 without sucrose; lane 3, prestained molecular weight markers (indicated on the left in kilodaltons); lane 4, MB2 with sucrose; lane 5, MB2 without sucrose; lane 6, WB30C with sucrose; lane 7, WB30C without sucrose. (D) Production of GroEL by DB104, MB2, and WB30C under heat shock conditions. Lane 1, WB30C without sucrose; lane 2, WB30C with sucrose; lane 3, MB2 without sucrose; lane 4, MB2 with sucrose; lane 5, WB30 without sucrose; lane 6, prestained molecular weight markers (indicated on the right in kilodaltons); lane 7, DB104 without sucrose.

ably GroEL) from *B. subtilis* which was also maximally synthesized at 10 min after heat shock (40). Characterization of the promoter region of the *B. subtilis groESL* operon should provide vital information for understanding how these genes are regulated under heat shock conditions. It is well established that the *E. coli rpoH* gene, which encodes a heat shock sigma factor, σ^{32}, is responsible for the heat-inducible transcription of most of the heat shock genes (12). Another novel sigma factor, σ^{E}, has also been identified from *E. coli* to transcribe the *rpoH* promoter at high temperatures (8). In *B. subtilis*, a minor sigma factor, σ^{D} (σ^{28}), has been implicated in transcribing heat shock genes, since this sigma factor can transcribe *E. coli* σ^{32}-specific promoters in vitro (4). However, disruption of the chromosomal *sigD* (structural gene for σ^{D}) demonstrated that this sigma factor is specific for the transcription of flagellar genes and possibly other chemotaxis genes (15). No functional role of *sigD* in the heat shock response has been established. In *B. subtilis*, it is interesting to note that the same *groESL* promoter is utilized under normal and heat shock conditions and that this promoter has consensus −35 and −10 regions similar to those recognized by σ^{A}-containing RNA polymerase. There are three inverted repeat sequences around the transcription start sites (two are downstream and one is upstream of the transcription start site). All of these inverted repeats are relatively short and A-T-rich sequences. No stable secondary structures are likely to be formed at 45°C under the reverse transcription condition, and thus, the transcription start sites determined by the primer extension analysis are unlikely to be an artifact due to the premature termination of the reverse transcription at the inverted repeat sequences. Several *B. subtilis* heat shock genes have been cloned and

sequenced. These include *grpE*, *dnaK*, and *dnaJ* (14, 46, 47). *grpE* and *dnaK* are likely to arrange in an operon, since the open reading frame for *dnaK* is 23 bp downstream from *grpE*. Although the promoter for these heat shock genes has not been mapped precisely, typical σ^A promoters can be found upstream of these genes. Thus, either the *B. subtilis* major sigma factor is responsible for transcribing these heat shock genes or an unidentified heat shock sigma factor which has almost the same recognition specificity as σ^A transcribes these genes. It has been demonstrated that the level of σ^A decreases during heat shock (1). A positive factor may be required to direct the σ^A-containing RNA polymerase to transcribe the heat shock genes (if σ^A-containing RNA polymerase is really responsible for the transcription of the heat shock promoters). The inverted repeat sequences around the *groESL* promoter can possibly serve as binding sites for this factor. Further characterization of the physiological role of these inverted repeats is in progress.

In *E. coli*, heat shock proteins such as DnaK, DnaJ, and GrpE have been suggested to function as a cellular thermometer (39). These proteins have a negative effect on the synthesis and stability of σ^{32}. Under heat shock, these proteins bind to the denatured or partially unfolded proteins. The reduction of the free pool of these proteins results in an increase of synthesis and stability of σ^{32}. A similar regulatory circuit may operate in *B. subtilis* if the GroES-GroEL complex exerts a negative effect on the hypothetical positive factor as discussed earlier. Other models that involve negative regulatory mechanisms (e.g., a temperature-sensitive repressor) cannot be excluded.

To determine whether *groES* and *groEL* are essential genes in *B. subtilis*, we had constructed a modified *groESL* operon by replacing the wild-type *groESL* promoter with the sucrose-inducible *sacB* promoter. This modified *groESL* operon was then applied to replace the chromosomal *groESL* through gene conversion, and the ability of the resulting mutant to grow under normal and heat shock conditions in the presence and absence of sucrose was monitored. There are two advantages using the *sacB* promoter for this study. (i) The *sacB* promoter is relatively weak, and thus, the basal expression level of *groESL* in the absence of inducer is undetectable. Consequently, the effect of depletion of GroES and GroEL on cell growth can be studied. (ii) The promoter strength of the *sacB* promoter can be regulated. The gene products of *degQ*, *degR*, and *sacU* are known to stimulate the transcription from the *sacB* promoter. With the construction of MB2 and WB30C [a *sacU*(Hy) strain carrying the modified *groESL* in the chromosome], the cellular level of GroES and GroEL can be varied by the addition of inducer, the use of a *sacU*(Hy) strain, or both. The direct correlation between the ability of these cells to grow under normal and heat shock conditions and the quantity of cellular GroEL (and possibly GroES) suggests the essential physiological functions of these proteins. This strategy can be applied to determine whether other known or novel genes are essential for normal cell growth. Recently, Wetztein and his coworkers (48) have independently cloned *groESL* from *B. subtilis*, and this operon was mapped to 342° on the *B. subtilis* genetic map. Further characterization of *groESL* would provide insights concerning its regulation and physiological roles under normal and heat shock conditions.

Alignment of various GroEL sequences (Fig. 3B) illustrates a striking feature of this protein family. A tandemly repeated Gly-Gly-Met motif was found at the carboxyl terminus. This sequence was repeated three times for the *B. subtilis* GroEL and four times for both the *E. coli* and *C. burnetii* homologs (Fig. 3). Similar GGM motifs were also observed for most of the eucaryotic Hsp60 homologs. Deletion of various length of the carboxyl terminus of yeast Hsp60 suggests that the presence of at least some (more than three) glycine and methionine residues is essential for cell viability (36). Cells which contain a 10-amino-acid extension at the carboxyl terminus of Hsp60 show a lower growth rate. A truncated version (minus GGM) of *E. coli groEL* was also constructed (27). The truncated protein hydrolyzes ATP 1.5 times more slowly than wild type and fails to suppress certain temperature-sensitive *dnaA* mutations. Construction of truncated *B. subtilis* GroEL with various deletions at the carboxyl terminus is in progress to examine the functional roles of this motif.

During the cloning of the *B. subtilis groESL* operon, the pGROESL plasmid was found to have four *Pst*I inserts. The 3.2-kb *groE* fragment alone could not be cloned into pBS or pUB18 without suffering rearrangement or deletion. It is observed that overproduction of GroES alone from high-copy-number plasmids causes toxicity to *E. coli* (53). However, overproduction of both GroES and GroEL or GroEL alone does not affect cell growth. It is proposed that GroES functions as a cogwheel in displacing the unfolded polypeptide which bound to the surface of GroEL oligomer (53). Overproduction of GroES would cause the premature release of these bound polypeptides and result in generation of misfolded and inactive proteins. Since the 3.2-kb *B. subtilis groE* fragment carries the entire *groES* and only part of *groEL*, the failure in cloning this fragment can be explained by the toxic effect of overproducing GroES. The insertion of other DNA fragments in pGROESL may reduce the copy number of the plasmid to the point that the cells can tolerate.

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