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Isolation and Characterization of *Bacillus subtilis groE* Regulatory Mutants: Evidence for *orf39* in the *dnaK* Operon as a Repressor Gene in Regulating the Expression of Both *groE* and *dnaK*

GANG YUAN AND SUI-LAM WONG*

Division of Cellular, Molecular and Microbial Biology, Department of Biological Sciences, University of Calgary, Calgary, Alberta T2N 1N4, Canada

Received 10 July 1995/Accepted 13 September 1995

An inverted repeat sequence known as CIRCE (controlling inverted repeat of chaperone expression) in the *Bacillus subtilis groE* operon has been suggested to function as an operator. To identify the regulatory gene directly or indirectly involved in CIRCE-mediated heat-inducible *groE* expression, *B. subtilis* WBG2, carrying an integrated *groE-bgaB* transcription fusion in the *amyE* locus, was mutagenized. Dark blue colonies formed at 37°C represent mutants which constitutively produce BgaB (a thermostable β-galactosidase) at high levels. Seven mutants (WBG101 to WBG107) were selected for further characterization. They all overproduced BgaB, GroEL, and DnaK simultaneously at 37°C. These mutants could be restored to normal by introducing a plasmid carrying a functional copy of *orf39*, the first gene in the *B. subtilis dnaK* operon. Genomic sequencing of these mutants demonstrated that they all carried a single mutation in *orf39*. These mutations can be divided into three groups: (i) Gly-307 to Asp, (ii) Ser-122 to Phe, and (iii) Gly-63 to Glu. By using a binary vector system in *E. coli*, production of ORF39 was found to negatively regulate the expression of *groE-bgaB* in a CIRCE-specific manner. Under the heat shock condition, the negative regulation mediated by ORF39 was abolished. Mobility shift of the CIRCE-containing probe was also observed with the crude extract prepared from the *E. coli* strain that overproduced ORF39. Therefore, ORF39 is the negative regulatory factor which regulates both *groE* and *dnaK* expression in *B. subtilis*. It is likely to function as a CIRCE-specific repressor.

Expression of *Bacillus subtilis* heat shock genes encoding molecular chaperones GroES, GroEL, and DnaK is regulated by a novel mechanism which differs from the well-characterized regulatory mechanism operating in *Escherichia coli*. In both the *B. subtilis groE* and *dnaK* operons, two sequence elements are found in the regulatory region. They are a typical σ^A-type promoter (16, 26, 33) and an inverted repeat (IR) sequence designated CIRCE (controlling IR of chaperone expression) (39). CIRCE is located downstream from the transcription start site in these operons. CIRCE-like IR sequences can also be found in *groE* or *dnaK* operons from many bacteria (28). Alignment of these sequences illustrates that a consensus sequence can be generated (20, 33).

For *groE*, identical transcription start sites are observed under both heat shock and non-heat shock conditions. Mutating either the first T nucleotide in the −10 region or the third G nucleotide in the −35 region of the *groE* promoter results in a significant decrease in promoter activity. The activities of these mutated promoters can be restored in an allele-specific manner by specific *sigA* mutants at both 37 and 48°C (37). Transcription studies of *groE* with the purified σ^A-containing RNA polymerase in vitro and a temperature-sensitive *sigA* mutant in vivo also support the involvement of the σ^A-containing RNA polymerase in transcribing *groE* (3). Since σ^A is the major sigma factor produced constitutively, some other mechanism(s) must be present to mediate the heat-inducible expression of these genes.

The CIRCE sequences in both the *B. subtilis groE* (37) and *dnaK* (39) operons are demonstrated to function as negative regulatory elements, since deletion or mutation of the IR sequence results in expression of these genes at high levels at 37°C. Transcripts of *groE* under heat shock and non-heat shock conditions are found to have the same in vivo half-life of 5 min, suggesting that CIRCE does not exert its negative effect by reducing the RNA stability. Furthermore, insertion of short sequences of 5 to 17 bp between the *groE* transcription start site and CIRCE results in a gradual abolishment of the negative effect mediated by CIRCE (37). CIRCE is therefore suggested to function as an operator. If this is the case, a repressor is expected to regulate the heat-induced expression of *groE* and *dnaK* operons. In support of this idea, a CIRCE-specific binding activity (5) is detected in both the crude extract and the partially purified fractions from *Streptomyces coelicolor* A3(2). To determine whether a repressor is involved in controlling *groE* expression, we report the development of a *B. subtilis* system to isolate regulatory mutants that affect both *groE* and *dnaK* expression and the characterization of these mutants. By using these mutants and a binary vector system in *E. coli*, *orf39* in the *dnaK* operon was shown to negatively regulate the heat-inducible expression of both *groE* and *dnaK* and is likely to encode a CIRCE-specific repressor.

MATERIALS AND METHODS

Bacterial strains and plasmids. *B. subtilis* 168 (*trpC2*) and *E. coli* DH5α [(φ80d*lacZ*Δ*M15*) *endA1 recA1 hsdR17* (r^- m^-) *supE44 thi-1* λ^- *gyrA relA1* F^- Δ(*lacZYA-argF*)*U169*] served as hosts for routine transformation. *B. subtilis* WBG2 (37) carrying the integrated *groE-bgaB* fusion (the inserted *groE* sequence containing both the *groE* promoter and CIRCE) was used for the mutagenesis experiment. Strain WBG3 (37) is identical to WBG2 except that only the *groE* promoter (without CIRCE) was included in constructing the integrated *groE-*

* Corresponding author. Mailing address: Department of Biological Sciences, University of Calgary, 2500 University Dr., NW, Calgary, Alberta T2N 1N4, Canada. Phone: (403) 220-5721. Fax: (403) 289-9311. Electronic mail address: slwong@acs.ucalgary.ca.

TABLE 1. Plasmid vectors used in this study

Vector	Host	Features	Reference
pUB18	*B. subtilis*	pUB110 derivative carrying the polylinker sequence from pUC18	31
pUB-P43	*B. subtilis*	Insertion of a 300-bp *Eco*RI-*Kpn*I fragment carrying the *B. subtilis* P43 promoter in pUB18	16
pUB-P43ORF39	*B. subtilis*	Insertion of a 1.1-kb *Kpn*I-*Xba*I fragment carrying *orf39* (nucleotides 221–1319 of the reported sequence) in pUB-P43. This insert was generated through PCR amplification. A *Kpn*I site and an *Xba*I site are added to the 5′ and 3′ ends of the fragment, respectively.	This work
pBS	*E. coli*	Bluescribe plasmid from Stratagene	
pDL	*E. coli*	*B. subtilis* integration vector with *bgaB* as the reporter gene	37
pDL-P43	*E. coli*	Insertion of a 300-bp *Eco*RI-*Kpn*I fragment carrying the *B. subtilis* P43 promoter in pDL	This work
pBS-P43ORF39	*E. coli*	Insertion of a 1.4-kb *Eco*RI-*Hin*dIII fragment carrying the P43-*orf39* expression cassette in pBS. This insert is from pUB-P43ORF39.	This work
pBS-MTL	*E. coli*	Insertion of a 1.5-kb *Hin*dIII-*Bgl*II fragment carrying the mannitol operon	This work
pET29bΔ(XB)	*E. coli*	pET29b from Novagen. Sequence from *Xba*I to *Bgl*II in pET29b was deleted through restriction digestion and end repairing (fill-in).	This work
pET-ORF39	*E. coli*	Insertion of a 1.1-kb *Kpn*I-*Hin*dIII promoterless *orf39* fragment from pBS-P43ORF39 in pET29bΔ(XB). Expression of *orf39* is controlled by a T7 promoter carrying the *lac* operator.	This work
pK184	*E. coli*	Cloning vector with the p15a replicon and a kanamycin resistance marker. This plasmid is compatible with pBS.	13
pK-BGAB	*E. coli*	Insertion of the 2.1-kb *Kpn*I-*Sst*I fragment carrying the *bgaB* reporter gene from pDL in pK184	This work
pK-GRO(+)	*E. coli*	Insertion of the 2.3-kb *Sst*I fragment carrying the *groE-bgaB* transcription fusion from pDL2 (37). The inserted *groE* regulatory region carries both the *groE* promoter and CIRCE.	This work
pK-GRO(−)	*E. coli*	Insertion of the 2.3-kb *Sst*I fragment carrying the *groE-bgaB* transcription fusion from pDL3 (37). The inserted *groE* regulatory region carries only the *groE* promoter. CIRCE is not included.	This work

bgaB fusion. This strain serves as the control for the mutagenesis experiment. *E. coli* BL21(DE3), obtained from Novagen, is a λDE3 lysogen of *E. coli* BL21 (F^- *ompT lonA* r_B^- m_B^-). This strain, which carries an integrated structural gene encoding T7 RNA polymerase, is used to express *B. subtilis orf39*. Plasmids used in this study are described in Table 1.

Culture conditions. Both *B. subtilis* and *E. coli* were cultured routinely in L broth. For heat shock studies, *B. subtilis* cells were cultured in L broth at 37°C up to 100 Klett units; cells were then transferred to 48°C for 30 min and collected for protein electrophoretic analyses. For the thermotolerance study, an overnight *B. subtilis* culture was transferred to four culture flasks containing fresh SMMYT medium (36) supplemented with 2% glucose. The initial cell density was adjusted to a value of 0.06 at A_{540}. Two sets of cells were cultured at 48°C. After a 30-min cultivation at this temperature, one set of the cultures was transferred to 52°C for the growth study and the other set was kept at 48°C. Two other cultures were cultivated directly at 37 and 52°C, respectively. For the study of the functional role of ORF39, *E. coli* cells carrying the appropriate plasmid(s) were cultured in L broth at either 37 or 42°C. For the one at 42°C, cells were cultured at this temperature for 15 min and then transferred to 48°C for 30 min. Cells were then collected to assay for the BgaB activity. To overproduce ORF39 for the band shift assay, *E. coli* BL21(DE3) carrying pET-ORF39 and the control strain *E. coli* BL21(DE3) carrying pET29bΔ(XB) were cultured in L broth containing kanamycin (30 μg/ml) at 37°C up to 100 Klett units. Isopropylthiogalactopyranoside (IPTG) at a final concentration of 0.4 mM was added to these cultures. Cells were cultivated for 2 h after induction and collected for the preparation of crude extracts.

Chemical mutagenesis. *B. subtilis* WBG2 was cultured in L broth (25 ml). Once the culture reached the exponential phase (optical density at 600 nm of 0.8), cells were harvested and washed once with prewarmed SC (0.15 M sodium chloride, 0.01 M sodium citrate [pH 7.0]). The cell pellet was resuspended in 10 ml of prewarmed SC containing *N*-methy-*N*′-nitro-*N*-nitrosoguanidine (NTG) at a final concentration of 100 μg/ml. Cells were cultured at 37°C with gentle shaking for 30 min. To stop the mutagenic reaction, cells were harvested, washed twice with L broth, and resuspended in 1 ml of L broth for a 1-h cultivation. The culture was collected and washed once with SC and resuspended in 10 ml of SC. Cells were then plated on tryptose blood agar base plates containing 5-bromo-5-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) and chloramphenicol (5 μg/ml).

Nucleic acid manipulation. Chromosomal DNA from *B. subtilis* was prepared as described previously (35). For genomic sequencing of the *groE* regulatory mutants, sequences of interest were generated through PCR amplification by using Vent polymerase (New England Biolabs Ltd., Canada) as recommended by the manufacturer. The amplified DNA samples were used directly as templates for sequencing by using the fmol DNA sequencing system (Promega, Wis.).

Band shift assays. *E. coli* BL21(DE3) carrying either pET29bΔ(XB) or pET-ORF39 was resuspended in cell breakage buffer (10 mM Tris-HCl [pH 8], 10 mM magnesium chloride, 0.1 mM EDTA, 1% glycerol, 1 mM dithiothreitol) and lysed by sonication. Cell debris and insoluble proteins were removed by centrifugation (12,000 × *g* for 5 min). The supernatant was collected for the band shift assay. The probe for the band shift assay was a 70-bp fragment carrying the *B. subtilis groE* regulatory sequence from −22 to +34 with the *Kpn*I site added at the 5′ end and the *Bam*HI site added at the 3′ end. This fragment was generated by PCR and end labeled by T4 polynucleotide kinase with [γ-^{32}P]ATP (35). In a binding assay reaction, the total volume for each assay was 30 μl. The binding mixture contained binding buffer (10 mM Tris-HCl [pH 7.8], 10 mM magnesium chloride, 100 mM sodium chloride, 2 mM dithiothreitol, 1% glycerol), 1 μg of sonicated salmon sperm DNA to reduce nonspecific binding, 0.2 pmol of the labeled CIRCE-containing probe, and 15 μg of total soluble protein from the *E. coli* crude extract. Binding was performed at 25°C for 15 min, and the samples were loaded to an 8% polyacrylamide gel, which run at 180 V (constant voltage) with 0.5× Tris-borate-EDTA buffer. To demonstrate that the binding was CIRCE specific, two assays were performed under special conditions. In one sample, 2 pmol of the nonlabeled CIRCE probe was added to the binding mixture containing all components except the labeled probe. After binding at 25°C for 10 min, the labeled probe (0.2 pmol) was added and the mixture was incubated at 25°C for another 10 min. The second control was prepared in the same manner except that the nonlabeled CIRCE probe was replaced by 4 μg of the nonlabeled sonicated salmon sperm DNA.

Other methods. BgaB activity was determined by a method described previously (37). The specific activity of BgaB is expressed as units per microgram of cellular protein. Antibodies against GroEL, DnaK, and BgaB were prepared in mice as described previously (16). Search for ORF39 homologs was performed through the electronic mail server, using the BLAST program from the National Center for Biotechnology Information and the Blitz program from EMBL. The ORF39 homologs were aligned with the Clustal program in the PC/GENE package.

RESULTS

Isolation of *B. subtilis* regulatory mutants affecting *groE* and *dnaK* expression. *B. subtilis* WBG2 (37) was mutagenized with NTG. In this strain, the *groE* regulatory region (from positions

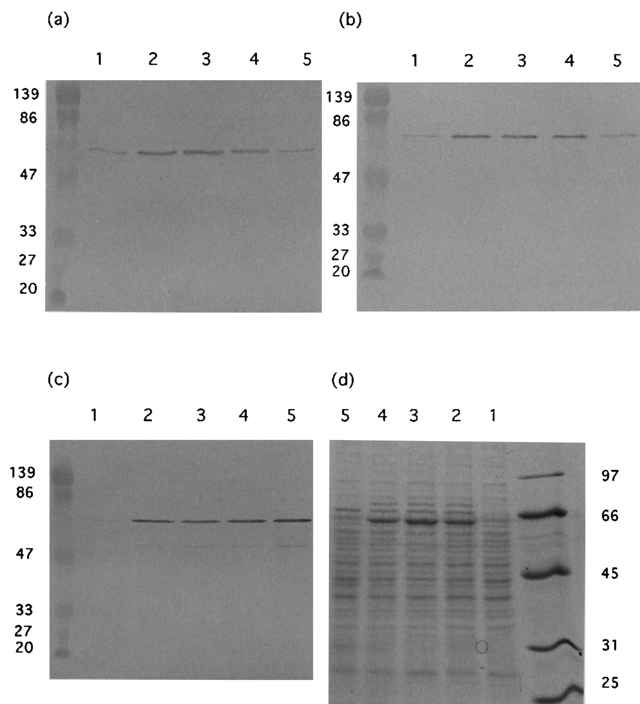

FIG. 1. Western blot and electrophoretic analyses of cellular proteins from various *groE* regulatory mutants. (a) Western blot with GroEL-specific antiserum; (b) Western blot with DnaK-specific antiserum; (c) Western blot with BgaB-specific antiserum; (d) SDS-polyacrylamide gel stained with Coomassie blue. Lanes: 1, *B. subtilis* WBG2; 2, *B. subtilis* WBG101; 3, *B. subtilis* WBG102; 4, *B. subtilis* WBG103; 5, *B. subtilis* WBG3. Sizes of protein molecular weight markers are indicated in kilodaltons.

−238 to +36, with +1 as the transcription start site) including the σ^A-type promoter and CIRCE was installed upstream of the promoterless *bgaB* gene encoding a thermostable β-galactosidase from *Bacillus stearothermophilus*. This *groE*(+IR)-*bgaB* transcription fusion was integrated at the *amyE* locus. At 37°C, colonies of WBG2 were white on the X-Gal-containing tryptose blood agar base plates. Under the heat shock condition, WBG2 colonies were blue. WBG3, a strain that is identical to WBG2 except that the CIRCE sequence was deleted in the *groE*(+IR)-*bgaB* transcription fusion, served as the control. Colonies of WBG3 showed a dark blue color when cultured at either 37 or 48°C. Inactivation of the repressor gene through mutagenesis should result in the constitutive expression of *groE*(+IR)-*bgaB*, *groE*, and *dnaK* simultaneously in WBG2 cultured at 37°C. When 5×10^5 NTG-treated WBG2 cells were plated on X-Gal-containing plates at 37°C, 17 colonies showed a dark blue color with the same intensity as that observed for WBG3. Many colonies with a frequency of 2/1,000 plated cells also showed a light blue color. Seven dark blue colonies were selected for further characterization and designated WBG101 to WBG107.

Characterization of the putative *groE* regulatory mutants. To confirm that these dark blue colonies represented the *groE* regulatory mutants, three of these mutants (WBG101 to WBG103) and the controls (WBG2 and WBG3) were cultured at 37°C, and their total protein profiles were analyzed by both sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and Western blot (immunoblot) analyses with individual BgaB-, GroEL-, and DnaK-specific antibodies (Fig. 1). Relative to the protein profile of WBG2 (lane 1), WBG3 (lane 5) showed an extra protein band with a molecular mass of 69 kDa. This band was confirmed to be BgaB by Western blotting (Fig. 1c, lane 5). WBG101 to WBG103 showed increased production of three proteins with molecular masses of 70, 69, and 66 kDa. They were confirmed to be DnaK, BgaB, and GroEL, respectively, by Western blot analyses (Fig. 1). The protein profiles from several light blue colonies were also determined. None of them showed the simultaneous overproduction of DnaK, BgaB, and GroEL (data not shown).

Nucleotide sequencing of the *groE* regulatory region in the *groE-bgaB* transcription fusion in WBG101. If the CIRCE sequences in *groE-bgaB*, *groE*, and *dnaK* are mutated simultaneously, it is possible to observe overproduction of all of the corresponding proteins under non-heat shock condition. To eliminate this possibility, the nucleotide sequence of the *groE* regulatory region (positions −238 to +36) including both the *groE* promoter and CIRCE from *groE-bgaB* in WBG101 was determined by direct genomic sequencing. No mutation was found in this region. Therefore, WBG101 represented a *groE* regulatory mutant.

Growth of WBG101 under various culture temperatures. Since at least two operons encoding molecular chaperones were expressed at high levels in WBG101, we wished to determine whether WBG101 can survive better than WBG2 under the heat shock condition. As shown in Fig. 2, WBG2 and WBG101 could grow equally well at 37 and 48°C. If both cell strains were heat shocked at a sublethal temperature (48°C)

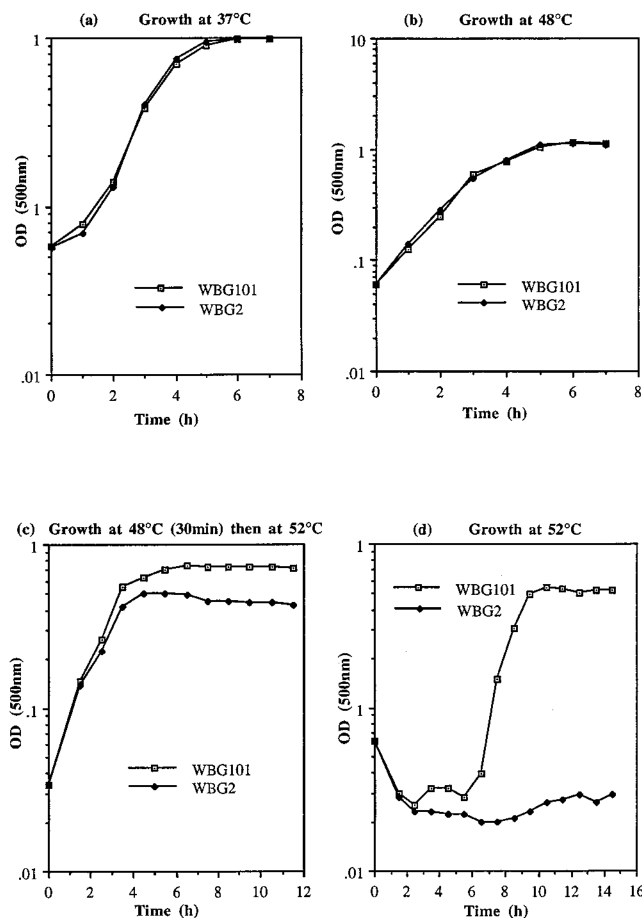

FIG. 2. Growth of the control strain (WBG2) and the mutant strain (WBG101) under various conditions. OD, optical density.

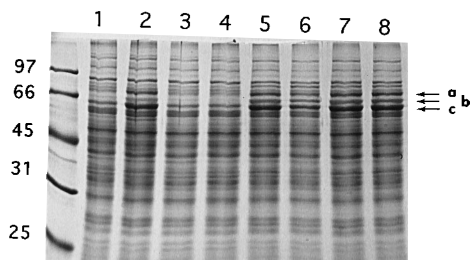

FIG. 3. Effects of expressing *orf39* in the *groE* regulatory mutant, WBG101, under heat shock and non-heat shock conditions on the production of GroEL, DnaK, and BgaB. Equal amounts of total intracellular proteins from WBG2 and WBG101 were analyzed on an SDS-polyacrylamide gel stained with Coomassie blue. Lanes 1 to 4 show the protein profiles from WBG2[pUB-P43], WBG101 [pUB-P43], WBG2[pUB-P43ORF39], and WBG101[pUB-P43ORF39], respectively. These four samples were collected from cells cultivated at 37°C. Lanes 5 to 8 show the protein profiles from the same series of cells cultivated at 48°C. a, b, and c represent DnaK, BgaB, and GroEL, respectively. Positions of the protein molecular weight markers are shown on the left.

for 30 min before being transferred to a lethal temperature (52°C), they all grew well. However, if both cell strains were transferred directly from 37 to 52°C, no growth of WBG2 could be observed for at least 15 h. WBG101 could adapt better and resume growth 7 h later after the temperature shift.

***orf39* in the *dnaK* operon can restore the *groE* regulatory mutants to normal.** While we were in the process of searching clones from a lambda phage library that can restore WBG2 to normal, a recent study by Schulz et al. (28) suggested that either *orf39* or *grpE* in the *dnaK* operon may be the regulatory gene controlling the expression of *groE*. Disruption of the entire *dnaK* operon, which encodes four genes in the order *orf39-grpE-dnaK-dnaJ*, results in expression of *groE* at higher levels, while inactivation of both *dnaK* and *dnaJ* does not affect the expression of *groE* (28). To test the idea that ORF39 is the key regulatory factor for *groE*, a 1.1-kb DNA fragment carrying the promoterless *orf39* (nucleotides 221 to 1319 of the reported *orf39* sequence [33]) was generated by PCR with the *B. subtilis* 168 chromosomal DNA as the template. This fragment was inserted in the expression vector pUB-P43. Expression of *orf39* was controlled by the constitutively expressed P43 (32) promoter. Mutant WBG101 carrying pUB-P43ORF39 reduced the expression of *groE*(+IR)-*bgaB*, *groE*, and *dnaK* to the background level (Fig. 3, lane 4). Expression of these genes at high levels could be observed when WBG101[pUB-P43ORF39] was heat shocked at 48°C (Fig. 3, lane 8). These results suggest that production of the functional ORF39 from the plasmid vector can restore WBG101 to the wild-type phenotype. Transformation of these *groE* regulatory mutants (WBG101 to WBG107) with the PCR-amplified, *orf39*-containing DNA fragment could also restore these mutants to normal.

Nature of the mutations in WBG101 to WBG107. To determine whether all seven mutants carry mutations in *orf39*, genomic sequencing was performed. Each mutant was found to have a single mutation in *orf39*. According to the location of the mutation, these mutants could be divided into three groups as shown in Table 2. Therefore, inactivation of *orf39* resulted in the constitutive expression of *gro*(+IR)-*bgaB*, *groE*, and *dnaK*. It is interesting that Gly-63 and Gly-307 are highly conserved among all currently reported ORF39 homologs (Fig. 4). These residues were changed to either Asp in the group 1 mutants or Glu in the group 3 mutant (Table 2). Besides the single mutation found in the reading frame of *orf39* in each mutant, all seven mutants consistently showed two discrepancies in sequence relative to the sequence reported previously (33). From this study, an extra G should be added between nucleotides 112 and 113 (GenBank accession number M84964), and a T at nucleotide 148 should be deleted. Both nucleotides were located upstream of the *dnaK* promoter. Genomic sequencing of this region from *B. subtilis* 168 indicated that *B. subtilis* 168 had the same sequence as observed in these mutants at these two positions. Therefore, discrepancies at these locations were not considered mutations.

TABLE 2. Mutations in WBG101 to WBG107

Mutant	Base substitution	Amino acid replacement
Group 1 (WBG 101, WBG104, WBG105)	GGC→GAC	Gly-307→Asp-307
Group 2 (WBG102, WBG106, WBG107)	TCC→TTC	Ser-122→Phe-122
Group 3 (WBG103)	GGA→GAA	Gly-63→Glu-63

Functional roles of ORF39. To examine whether ORF39 functions as a repressor, *E. coli* was selected as the host for this study. Since expression of heat shock genes in *E. coli* is not

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ORF39BS     M---LTNRQLL----------ILQVIINDFIKSAQPVGSRTLSKKDEITF     37
ORF39CA     ME--MEERKLK----------ILQAIINDYINNGEPVGSRTIAKKYNLGI     38
ORF37SA     M---ITDRQLS----------ILNAIVEDYVDFGQPVGSKTLIERHNLNV     37
ORF39LL     M---ITERQRQ----------ILNLIVSLYAKDHTPIGSKSLLD--SIQA     35
ORF45CT     MENRIEMSQLRASKKDSKISYVLLMATKLYLESGQPVGSKLLKETYCSDL     50
            *    .. ..           .*   .. . .   *.**. . .

ORF39BS     SSATIRNEMADLEELGFIEKTHSSSGRVPSEKGYRYYVDHLLSPVKLTKS     87
ORF39CA     SSATIRNEMADLEEMGYIEQLHTSSGRKPSDKGYRLYVDRLMEIPSMSVE     88
ORF37SA     SPATIRNEMKQLEDLNYIEKTHSSSGRSPSQLGFRYYVNRLLEQTSHQKT     87
ORF39LL     SSATIRNDMKALERLGLIQKEHTSSGRIPSVSGYKYFVENVIQLEEFSQN     85
ORF45CT     SSATIRNYFAQLETDGFLRKNHISGGRIPTDLAFRYYADH--NAPFLEQE     98
            *.***** . .** . . . * *.** *.  ... ....         .

ORF39BS     DLDQIHS-IFKEKIF--ELEKTVQKSAQILSDLTNYTSIVLGPKLSENYL    134
ORF39CA     EEMLIKAKIIDSALY--EIDKLVKQAMSLVSEMTKLTCVVKSLSARKSYI    136
ORF37SA     NKLRRLNQLLVENQY--DVSSALTYFADELSNISQYTTLVVHPNHKQDII    135
ORF39LL     DLFKVM-KAFDGDFY--RLSDLFKTAAKSLSELTGLTSFVLNAPQRDQQL    132
ORF45CT     EILAIQQKLTELPEYSKNIVKDLQKASEVLSDILQLPVCFSSPRFESDSV    148
            .       .  . .         .*..   .         .

ORF39BS     KQIQIIPIQPDMAVAILVTNTGHVENKTINFPTKMDLSDIEKLVNILNDR    184
ORF39CA     KSISLINIEPNMILCVFITDSGMIKNSIIRVKSNIENSSLERIANILNSK    186
ORF37SA     NNVHLIRANPNLVIMVIVFSSGHVEHVHLASDIPFSNDKLNTISNFVTNK    185
ORF39LL     VSFEMVMLDNHSVLSVITLGTGEVRTNQFILPKSMTEADLAVFSNLVKER    182
ORF45CT     INIQLVAIDDQRVVFVLSTEFGQVFTDVLWLPEQLPENSLKRIEGFLQNY    198
                ..  . .  . ..  . * .   .     .  ...  . ... .

ORF39BS     LSGVPMDELNERIFKEVVMYLRQHIKNYDNILDALRSTF--HSTNHVEKL    232
ORF39CA     LKGLTIEQINLEVINNIKKDLREYGHIFDCIMPNLYDIL--READSTE-V    233
ORF37SA     LT-----EFNQNLQDDIVSFVQSEQE--EIFINKLINTMNNHISNQSNSI    228
ORF39LL     LVGKKVIDIHYTLRTEIPQIVQRYFKVTSEVLQLFESIFDDLFKEH---L    229
ORF45CT     LRKQPSDSLLSQKEEDLGMVL------YNEVVVRYLTRYCHFSEED---L    239
            *       .    ...  .      .  .   .       .  .

ORF39BS     FFGGKINMLNQPEFHDITRVRSLLSLIEKEQ--DVLKLVQSPHTGISIKI    280
ORF39CA     YKEGTMNIFNYPEFKDIEKAKEFLSVID-DR--RILDTLFNASGGVTVNI    280
ORF37SA     YMGGKVKLIDALNESNVSSIQPILQYIESNRIAELLQDISSPN--INVKI    276
ORF39LL     TVAGHKNIFDYAT-DNLAELYKLFS--DDERMLHEIREITNNDEMRAVKF    276
ORF45CT     YQTGLSRLLKYETFKDPETLAQGLAFFENRKHMCQLLNTYLHKETPTAFI    289
              .*  ....  .   . .     .    .   .    .          .  .

ORF39BS     GKENDY---EEMENCSLITASYSVDQKQIGSIAIIGPTRMNYSRVVSLLQ    327
ORF39CA     GNENSI---KEARDFSVVSSVYKYNGRPLGTIGIIGPTRIPYSKVIKVIM    327
ORF37SA     GNEID----DSLSDISIVTSQYHFDETLKGQIAVIGPTAMHYQNVIQLLN    322
ORF39LL     DNDEKF-----MKNLTIISQKFVIPYRGFGTLTVVGPVEMDYQRTLSVLD    321
ORF45CT     GRELTDIVGNTDPSCAVITIPYYMDRTPLGTFGVLGPMNLPYQQVFGTLS    339
            ... .          . ....  .        * ....**  . * ..   .

ORF39BS     HVTSDLSKALTS-LY-----------------------DE----------    343
ORF39CA     EVVDQINNNLDK-MN-----------------------NS----------    343
ORF37SA     RI-------------------------------------W----------    325
ORF39LL     LVAKVLTMKLSD-YYRYL-------------DGNHYEISK----------    347
ORF45CT     LFTERLKVILTQSFYKFKLSFRRPCPTDPRCSQRPAELTRSSSIKLLPAK    389

ORF39BS     ---    343
ORF39CA     ---    343
ORF37SA     ---    325
ORF39LL     ---    347
ORF45CT     ELS    392
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FIG. 4. Sequence alignment of *B. subtilis* ORF39 with ORF39 homologs from other microorganisms. Identical and conserved residues are marked with asterisks and dots, respectively. Sequences in the highly conserved region are shown in boldface. Amino acid residues that were changed in the *B. subtilis groE* regulatory mutants are shown in italic. BS, *B. subtilis*; CA, *Clostridium acetobutylicum*; SA, *Staphylococcus aureus*; LL, *Lactococcus lactis*; CT, *Chlamydia trachomatis*.

TABLE 3. Use of a binary vector system to study the regulation of *groE-bgaB* expression by ORF39 in *E. coli*

Vector 1	Vector 2	BgaB sp act[a]	
		37°C	48°C
pBS-ORF39	pK-BGAB	7.9	7.5
pBS-ORF39	pK-GRO(−)	2,041	1,884
pBS-ORF39	pK-GRO(+)	135	728
pBS-MTL	pK-BGAB	3.1	4.2
pBS-MTL	pK-GRO(−)	1,862	1,992
pBS-MTL	pK-GRO(+)	1,904	2,006

[a] Average of three independent experiments; expressed as units per microgram of cellular protein.

regulated by the CIRCE-dependent mechanism, the CIRCE-specific repressor should not exist in this host. By the introduction of the P43-*orf39* cassette in pBS, a high-copy-number plasmid, and the *groE-bgaB* transcription fusion in pK184, a low-copy-number plasmid with the p15a replicon, regulation of *bgaB* expression by *orf39* under both heat shock and non-heat shock conditions was examined (Table 3). If ORF39 functions as a repressor, overproduction of ORF39 from the high-copy-number plasmid should shut off the expression of *groE-bgaB* from the low-copy-number plasmid. SDS-PAGE analysis indicated that *E. coli* DH5α carried either pBS-P43ORF39 alone or pBS-P43ORF39 in combination with the pK184 derivative produced ORF39 at high levels (data not shown). Although the majority of the overproduced ORF39 formed inclusion bodies, there was sufficient soluble ORF39 to mediate the expression of *groE-bgaB*. As shown in Table 3, DH5α[pBS-P43ORF39, pK-BGAB] under both heat shock and non-heat shock conditions showed a background level of *bgaB* specific activity (7 U/μg of protein) from the promoterless *bgaB* reporter gene. With the insertion of the *groE* promoter alone (without CIRCE) to *bgaB*, DH5α[pBS-P43ORF39, pK-GRO (−)] showed high levels of BgaB activity regardless of the cultivation temperature (37 and 48°C). However, if the *groE* regulatory region including both the promoter and CIRCE was inserted into *bgaB*, BgaB activity from DH5α[pBS-P43ORF39, pK-GRO(+)] cultured at 37°C decreased 15-fold. This decrease in BgaB activity depended on the presence of plasmid pBS-P43ORF39. Upon replacement of pBS-P43ORF39 by pBS-MTL, a pBS plasmid carrying a fragment of the mannitol operon, BgaB activity in DH5α[pBS-MTL, pK-GRO(+)] remained at high levels. These data illustrated that ORF39 negatively regulated the expression of *groE* and that this regulation was CIRCE specific. It is important to note that when DH5α [pBS-P43ORF39, pK-GRO(+)] was exposed to a heat treatment, a fivefold increase in BgaB specific activity was observed. This observation suggested that ORF39 could be heat inactivated either directly (e.g., ORF39 is a temperature-sensitive repressor) or indirectly (e.g., possibly through proteolytic degradation by heat-inducible proteases such as Lon or by chemical modifications).

Detection of CIRCE-specific binding activity. To strengthen the idea that ORF39 is a CIRCE-specific repressor, crude extracts were prepared from both the control strain [*E. coli* BL21(DE3) carrying pET29bΔ(XB)] and the ORF39 production strain [*E. coli* BL21(DE3) carrying pET-ORF39] for the mobility shift assay. Expression of *orf39* in this system was controlled by the IPTG-inducible T7 promoter. With the production of ORF39 at high levels, a low percentage of ORF39 was in the soluble form (data not shown). As shown in Fig. 5, the CIRCE-containing probe was shifted to a new position only by the extract prepared from the ORF39 production strain (lane 3). Prebinding of the extract with the nonlabeled probe (2 pmol) effectively abolished the observed mobility shift of the labeled probe (lane 4). However, prebinding with 4 μg of sonicated salmon sperm DNA did not significantly affect the interaction of the extract with the labeled probe. These observations indicated the binding activity in the ORF39-containing extract was CIRCE specific.

DISCUSSION

To isolate the *groE* regulatory mutants in the genetic background of WBG2, two important selection criteria were used in the screening process. These mutants should show dark blue colonies with the same degree of color intensity as that observed for WBG3, a strain that is identical to WBG2 except that the CIRCE sequence in the *groE*(+IR)-*bgaB* transcription fusion is deleted. This criterion allows the screening of mutants with full inactivation of the *groE* repressor activity. During the screening process, many light blue colonies were observed. None of them showed a simultaneous overproduction of all three markers. Under the following four situations, light blue colonies can possibly be detected: (i) mutations in either *B. subtilis lacA*, which encodes β-galactosidase, or its regulatory gene (*lacR*) for the putative repressor (7); (ii) partial inactivation of the *groE*/*dnaK* repressor; (iii) mutations in the CIRCE sequence of the *groE*(+IR)-*bgaB* fusion (these mutations can be located at positions that weakly affect the binding of the *groE*/*dnaK* repressor); and (iv) mutations affecting the proper folding of certain cellular proteins, which may lead to increased *groE* expression.

The second selection criterion is that at least three proteins, the 70-kDa DnaK, the 69-kDa BgaB, and the 66-kDa GroEL, should be overproduced simultaneously in these mutants when they are cultured at 37°C. This will eliminate the possibility that these dark blue colonies are formed because of mutations in the CIRCE sequence in the *groE*(+IR)-*bgaB* transcription fusion.

Isolation of seven dark blue colonies (WBG101 to WBG107) provides evidence to support the idea that a common negative regulator plays a vital role in regulating the heat-inducible expression of both *groE* and *dnaK* operons in *B. subtilis*. The ability to restore these mutants to normal by providing the P43-*orf39* cassette on pUB18 (Table 3) and determination of the nature of the mutations in these strains (Table 2) demon-

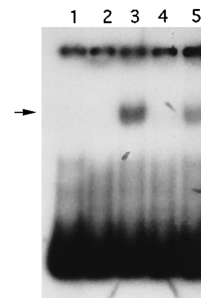

FIG. 5. Band shift assays of the CIRCE probe with crude extracts prepared from *E. coli*. The radioactive bands at the top and bottom of the gel represent radioactive material retained the top of the wells and the labeled probe, respectively. The protein-probe complex is marked by an arrow. Lanes: 1, the labeled CIRCE probe alone without addition of any crude extract; 2, crude extract from the control strain *E. coli* BL21(DE3)[pET-29bΔ(XB)] added; 3, extract from *E. coli* BL21(DE3)[pET-ORF39] added; 4, same as lane 3 except that 2 pmol of the unlabeled CIRCE probe was added for prebinding; 5, same as lane 3 except that 4 μg of sonicated salmon sperm DNA was added for prebinding.

strate that *orf39*, the first gene in the *dnaK* operon (33), encodes the negative regulatory factor for controlling the expression of *groE* and *dnaK* operons.

ORF39 can act either directly as a repressor which binds specifically to CIRCE or indirectly as an activator which leads to the production of the functional repressor. For the activator model, ORF39 can be either a transcription activator required for the expression of the *groE/dnaK* repressor or a specific modifying/processing enzyme required for the generation of the active *groE/dnaK* repressor. Alternatively, it can be a molecular chaperone to mediate the proper folding of the repressor under the non-heat shock condition. Our data (Table 3) favor the repressor model. If ORF39 acts indirectly, a structural gene encoding the CIRCE-specific repressor should exist in *B. subtilis*. This repressor gene is unlikely to be present in *E. coli*, since it is well established that heat shock regulation in *E. coli* is mainly mediated through two alternate sigma factors, σ^{32} (10, 29) and σ^{E} (11, 18, 24, 25). Up to now, neither the CIRCE-like sequence nor the ORF39 homolog has been found in *E. coli*.

If ORF39 is the repressor, all other microorganisms with their molecular chaperone genes regulated through the CIRCE-specific mechanism should have an ORF39 homolog. Although the *dnaK* operons in many microorganisms are not completely sequenced, four microorganisms (6, 21, 22), *Clostridium acetobutylicum*, *Staphylococcus aureus*, *Lactococcus lactis*, and *Chlamydia trachomatis*, with CIRCE sequences in their *dnaK* operons were found to carry a homologous *orf39* gene. As shown in Fig. 4, amino acid sequence alignment illustrates that two regions are more conserved. By using the *B. subtilis* ORF39 as a reference, these conserved regions are located at positions 9 to 77 (the N-terminal region) and positions 307 to 322 (the C-terminal region), respectively. The change of the highly conserved Gly-307 to Asp in the group 1 mutants and Gly-63 to Glu in the group 3 mutant resulted in the inactivation of ORF39. These glycine residues are likely either structurally or functionally important. Since this group of proteins is predicted to bind to CIRCE, the conserved N-terminal region may serve as the CIRCE binding domain.

orf39 is the first gene in the *B. subtilis dnaK* operon. Its regulatory effect should result in a negative autoregulation of its own expression. This prediction is consistent with the observation from the titration experiment. CIRCE was inserted in pUB18 (a high-copy-number plasmid), and the CIRCE-containing plasmid (pUB-CIRCE) was transformed to WBG2. If the repressor is not negatively autoregulated, titration of the repressor by pUB-CIRCE should increase the expression of the chromosomal *groE*(+IR)-*bgaB*. However, we failed to observe any significant increase in BgaB activity (data not shown). This negative autoregulation may be a mechanism to ensure a low basal level of these molecular chaperones in the cell under the non-heat shock condition.

Under the heat-shock condition, up to 66 heat shock proteins can be detected in *B. subtilis* (1, 19, 30). Among these heat shock proteins, the functional roles for GroES/GroEL, DnaK, DnaJ, and GrpE in facilitating protein folding (15, 17), assembly (2, 8, 38), and export (14, 23, 34) and in minimizing protein aggregation (4, 9, 12, 27) are well established. As shown in Fig. 3, the production levels of GroEL and DnaK in WBG101 cultivated at 37°C (lane 2) are comparable with those in WBG2 cultivated at 48°C (lane 5). With the constitutive expression of both *groE* and *dnaK* operons in WBG101, this mutant can recover faster and regain the ability to grow within 7 h after heat shock at a temperature that is lethal to wild-type *B. subtilis*. As a control, WBG2 was unable to grow under the same condition (Fig. 2). These data reflect the importance of these five molecular chaperones for cell survival under high temperatures. However, other heat shock proteins are clearly required for rapid adaptation, since a sublethal heat treatment at 48°C for 30 min, which induces the expression of all heat shock genes, allows rapid growth of *B. subtilis* at 52°C.

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