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The Response of a *Bacillus subtilis* Temperature-Sensitive *sigA* Mutant to Heat Stress

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The mutant *sigA* allele of *Bacillus subtilis* DB1005 was confirmed to be temperature sensitive (*ts*) and transferable among strains of *B. subtilis* by chromosomal transformation and gene conversion. This *ts sigA* allele had a pleiotropic effect on gene expression of DB1005. The induction of certain heat shock proteins in DB1005 was markedly less significant than that observed in the wild-type strain (DB2) under heat stress. In contrast, some proteins required for coping with oxidative stress and glucose starvation were induced abruptly in DB1005 but not in DB2. Heat induction of the *groEL* gene in vivo at both transcription and translation levels was much lower in DB1005 than in DB2. Besides, the putative σ^A-type promoter from the *groESL* operon of *B. subtilis* was able to be transcribed by the reconstituted σ^A RNA polymerase in vitro at both 37 and 49°C. These results strongly suggest that the expression of the *groEL* gene of *B. subtilis* under heat stress is regulated at least in part by σ^A at the level of transcription. Our results also showed that DB1005 did not respond too differently from the wild type to ethanol stress, except after a relatively long exposure.

σ^A factor of *Bacillus subtilis* plays important roles both in the maintenance of vegetative growth and in the regulation of sporulation. To study the structural and functional properties as well as the roles of σ^A in the regulation of *B. subtilis* cell development, we constructed a temperature-sensitive (*ts*) *sigA* mutant named DB1005. Our data showed that the temperature sensitivity of this mutant did not result from a rapid degradation of the *ts* σ^A protein (8); other causes should be responsible for the defect. Therefore, the characterization of this mutant under heat stress becomes our main interest for the moment.

It is known that a characteristic set of proteins is induced in nearly all organisms under heat shock and other forms of environmental stress (26, 27, 33). In *Escherichia coli*, the heat shock protein genes are under the control of a minor sigma factor, σ^{32} (18, 43). The heat shock response of *B. subtilis* in several strains of bacilli has been studied (1, 30, 44); however, the mechanism of its regulation remains unclear. The σ^{28} RNA polymerase was once thought to transcribe the heat shock genes of *B. subtilis* because of its overlapping promoter specificity with *E. coli* σ^{32} RNA polymerase (2). However, experiments which disrupted the structural gene of σ^{28} demonstrated that this σ affected only the transcription of flagellar and possibly other chemotaxis genes (20) but had nothing to do with heat shock. Recent studies indicated that the transcription starting sites of certain heat shock protein genes (*groESL* operon and *dnaK* locus) of *B. subtilis* were preceded by a σ^A-type promoter, and no other promoter sequences recognizable by other sigma factors were identified (25, 47). Thus, it is likely that σ^A itself or σ^A in accompaniment with other regulatory elements is involved in the regulation of *B. subtilis* heat shock response. This conception was put to the test in vivo by employing the *ts sigA* mutant and in vitro by transcription with reconstituted σ^A RNA polymerase holoenzyme, and the outcome was fruitful.

In this paper we report the responses of DB1005 to the elevation of temperature and the treatment of ethanol. Rates of total RNA and protein synthesis as well as expression of the *groEL* gene in both mutant and wild-type cells under heat stress are also compared. Our results indicate that the *ts sigA* allele has a pleiotropic effect on the expression of genes in DB1005 under heat stress. More importantly, the *groEL* gene of *B. subtilis* is regulated at least in part by σ^A at the level of transcription.

MATERIALS AND METHODS

Enzymes and chemicals. Restriction enzymes, ligase, and other DNA modification enzymes were purchased from Bethesda Research Laboratories and used according to the recommendations of the manufacturer. L-[^{35}S]methionine (1,200 Ci/mmol), L-[4,5-^{3}H]leucine (20 to 50 Ci/mmol), [5,6-^{3}H]uridine (35 to 50 Ci/mmol), and [α-^{32}P]dCTP (3,000 Ci/mmol) were obtained from Amersham Corp. Ampholites for isoelectric focusing (IEF) gels were purchased from Serva. A random priming kit for DNA probe preparation was ordered from Boehringer Mannheim. Other reagents were products of Bio-Rad Laboratories and Merck.

Bacteria and plasmids. *B. subtilis* strains including DB1005 [*trpC sigA* (Ts)] and DB403 (*trpC npr aprE epr*) are all derivatives of DB2 (*B. subtilis* 168 *trpC*). Plasmids and *E. coli* strains used for the overexpression of GroEL protein were the same as previously reported (6, 37). The pYJ12 plasmid used for gene conversion experiments was a derivative of a promoter probing plasmid pWP18 (46). To construct pYJ12, a *Sal*I DNA fragment encompassing the *apr* gene in pWP18 was first removed to form pYJ2. An N-terminally truncated wild-type *sigA* gene (without a ribosome binding site) was then prepared by digestion of a PCR-amplified *sigA* DNA with *Bam*HI, and this *Bam*HI DNA fragment was cloned into pYJ2 to form the plasmid pYJ12. Thus, there was no promoter and ribosome binding site upstream of the *sigA* gene.

Total RNA and protein synthesis. The glucose minimal medium (42) supplemented with 0.004% tryptophan was used throughout the whole experiment. Cells grown in this medium were pulse-labeled for 1 min, at designated times, by adding 200 μl of the same medium containing either 0.8 μCi of

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[^{3}H]uridine (for RNA determination) or 0.8 μCi of [^{3}H] leucine (for protein determination) to 200 μl of cultures which had been transferred from 37 to 49°C at an optical density (A_{550}) of 0.4. Incorporation was terminated by adding an equal volume of 10% trichloroacetic acid to the labeled cultures. Samples were then filtered onto a GF/C disc and counted by a Packard scintillation counter.

Pulse-labeling and analysis of the heat shock proteins. Methods for pulse-labeling and analysis of the heat shock proteins by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) were the same as those described in a previous paper (1). Two-dimensional (2-D) gel electrophoresis of the heat shock proteins was based on the method of O'Farrell (34). Sample preparation and conditions for 2-D analyses were as follows: after a pulse-labeling of the sample cells with [^{35}S]methionine, 0.8 ml of the labeled culture was harvested by centrifugation (4°C, 12,000 × *g*, and 10 min) and washed twice with cold medium. The resulting pellet was then treated with 15 μl of lysing solution (1 mg of lysozyme per ml, 0.5 mg of DNase per ml, 0.5 mg of RNase A per ml, 2 mM phenylmethylsulfonyl fluoride) for 5 min at 37°C and disrupted with 15 μl of a solution comprising 4% SDS and 5% 2-mercaptoethanol. The sample was heated at 90°C for 3 min, its radioactivity was determined, and 10 μl of this sample was mixed with 30 μl of sample buffer (9.5 M urea, 1.6% Servalyte [pH 5 to 7], 0.4% Servalyte [pH 3 to 10], 2% Nonidet P-40, 0.7 M 2-mercaptoethanol) before being loaded on an IEF gel. The IEF gels were prerun at 250, 500, and 700 V for 30 min for each voltage step, protein samples were loaded on the basic ends of the gels, each sample was overlain with 15 μl of overlay buffer (4 M urea, 0.8% Servalyte [pH 5 to 7], 0.2% Servalyte [pH 3 to 10]), and the IEF gels were run at 700 V for 18 h. Following IEF, each IEF gel was soaked in 40 ml of stacking buffer (0.126 M Tris-HCl [pH 6.8], 0.1% SDS) for 40 min, and each gel was placed on top of a slab gel and sealed with 1% agarose in stacking buffer. After the 2-D electrophoresis, the slab gels were stained with Coomassie blue to locate the protein standards and exposed to X-ray film after being dried.

Immunoblot analysis of σ^A and GroEL protein. The method for immunoblot analysis of GroEL and σ^A proteins was the same as that previously reported (6). Antibody to σ^A protein was prepared as mentioned in our previous report (7). To prepare the antibody for *B. subtilis* GroEL protein, two primers (5′-CTGAGGATCCGCTGTTATCGGCTAATTC-3′ and 5′-CTGAGGATCCGACGGTTAAAACATTGATG-3′) for the *groEL* gene were synthesized on the basis of its DNA sequence (25, 39) and were used to amplify the *groEL* DNA from the *B. subtilis* DB2 chromosome by PCR. The amplified *groEL* gene was subsequently digested with *Bam*HI and cloned into an overexpression vector (37) to overproduce the GroEL protein. The overexpressed GroEL protein was then cut out from the SDS-polyacrylamide gel and recovered by electroelution. About 400 μg of the purified GroEL protein was mixed with Freund's complete adjuvant and injected into a New Zealand White rabbit. Two and 4 weeks later, the rabbit was boosted with another 200 μg of the GroEL protein. Antibody obtained by this procedure was pure enough for the detection of *B. subtilis* GroEL protein as a single band on SDS-polyacrylamide gels.

RNA extraction and Northern blot analysis of *groEL* mRNA. The method for RNA extraction from *B. subtilis* was as follows: 10 ml of *B. subtilis* culture was pelleted by centrifugation for 10 min at 4°C, washed with 0.5 ml of SET buffer (20% sucrose, 50 mM Tris-HCl [pH 7.6], 50 mM EDTA), and resuspended in 1 ml of the same buffer containing 2 mg of lysozyme per ml. The suspension was further incubated at 37°C for about 5 min and centrifuged at 4°C for 5 min to collect the soft cell pellet; 0.5 ml of hot (60°C) denaturation buffer (4 M guanidium-thiocyanate, 50 mM Tris-HCl [pH 7.6], 10 mM EDTA, 2% SDS, 0.14 M 2-mercaptoethanol) and 50 μl of 2 M sodium acetate (pH 4.0) were then added to the pellet. The cell suspension was vortexed for 10 s before 0.5 ml of hot phenol (saturated with diethylpyrocarbonate-treated water) and 0.2 ml of chloroform-isoamyl alcohol (24:1) were added. After the addition of phenol and chloroform, the mixture was vortexed and put in an ice bath for 15 min. The mixture was centrifuged for 5 min at 4°C, and the supernatant was saved. An equal volume of isopropanol was added to the supernatant, and the solution was kept at −20°C for at least 1 h before the RNA was pelleted. The RNA pellet was dissolved in 0.3 ml of denaturation solution and precipitated again by addition of the same volume of isopropanol. The solution was kept at −20°C for another 1 h, and the pellet was centrifuged down at 4°C for 15 min. After the RNA pellet was washed with 70% ethanol, the pellet was dried and resuspended in 200 μl of TEN buffer (10 mM Tris-HCl [pH 8.0], 10 mM NaCl, 1 mM EDTA) containing 0.1 mg of proteinase K per ml. The solution was incubated at 37°C for 1 h and extracted twice with phenol-chloroform. The RNA was pelleted again and resuspended in 100 μl of DNase I solution (40 mM Tris-HCl [pH 8.0], 6 mM $MgCl_2$, 10 mM NaCl) containing 30 U of DNase I. The solution was incubated at 37°C for 1 h and extracted twice with phenol-chloroform. After further precipitation, the RNA pellet was dissolved in water treated with diethylpyrocarbonate. RNA was quantitated by determining its optical density at 260 nm. Northern (RNA) blot analysis was carried out as described elsewhere (38).

N-terminal sequencing of proteins. Protein samples for N-terminal sequencing were prepared in the following manner. Forty milliliters of *B. subtilis* culture grown in glucose minimal medium was pelleted and resuspended in 0.7 ml of extraction buffer (0.7 M sucrose, 0.5 M Tris-HCl, 30 mM HCl, 50 mM EDTA, 0.1 M KCl, 2% 2-mercaptoethanol, 2 mM phenylmethylsulfonyl fluoride). The resuspended cells were sonicated before an equal volume of water-saturated phenol was added, and the mixture was vortexed vigorously for 1 min. After the mixture was spun at room temperature for 10 min, the phenol phase was saved and extracted with extraction buffer to remove residual nucleic acids. Five volumes of 0.1 M ammonium acetate-methanol solution was then added to the phenol phase, and the resulting mixture was kept at −20°C for 2 h. The mixture was centrifuged at 12,000 × *g* and 4°C for 10 min to collect the protein pellet, and the pellet was washed twice with 1 ml of 0.1 M ammonium acetate-methanol solution. The protein pellet was further vacuum dried and used for 2-D gel electrophoresis separation as well as isolation of the desired proteins. The isolated proteins were finally transferred to the polyvinyl difluoride membrane and subjected to N-terminal sequencing by an amino acid sequencer (ABI model 476) at the Agricultural Biotechnology Laboratories, National Chung-Hsing University.

In vitro transcription with reconstituted RNA polymerase holoenzyme containing σ^A. Core enzyme and σ^A were prepared as described previously (6, 10, 14, 19). Procedures for RNA polymerase holoenzyme reconstitution and in vitro transcription were modified from a previous method (6). In this study, the mixture of reconstituted RNA polymerase holoenzyme and promoter DNA was incubated at 37°C for 5 min before the reaction cocktail, which was prewarmed at 37 or 49°C, was added. After the addition of reaction cocktail, in vitro transcriptions at both 37 and 49°C were performed. Promoter DNA templates required for in vitro transcription

were prepared by PCR. Nucleotide sequences of primers for PCR were as follows: the forward and reverse primers for the putative σ^A-type promoter from the *B. subtilis groESL* operon were 5′-CTGAGGATCCTCCGGCATAACCGAAT-3′ and 5′-CTGAGGATCCGAATTAGCCGATAACAGC-3′, respectively. Those for the P3 promoter of the *B. subtilis sigA* operon were 5′-GTCGGGATCCCGGAAATTTTTCGAC-3′ and 5′-GTGTCGAAGCTTCTTTATGAGGA-3′, respectively. Primers for the ϕ29 phage G3aG3b tandem promoter (11) were mentioned previously (7). The *groESL* promoter DNA was 651 bp long and had a transcript of 363 bases in length. The G3aG3b promoter, which was 315 bp in length, had a transcript of 142 bases starting from the G3b promoter. In addition, the P3 promoter DNA was 285 bp in length, and the transcript from the starting site of this promoter was composed of 178 bases.

RESULTS

Identification of the *ts sigA* allele. To ascertain that the intact *sigA* allele of *B. subtilis* DB1005 was responsible for the temperature sensitivity of the cells, chromosomal DNA transformation and gene conversion (4, 21, 22) experiments were performed. In the former experiment, chromosomal DNA extracted from the *ts* mutant was transformed into DB403 which contained a wild-type *sigA* gene. Since a *cat* gene has been integrated into the chromosome of DB1005 in the vicinity of *sigA* (about 1.5 kb apart) (8) during the construction of this mutant, we expected to see a high cotransformation efficiency of chloramphenicol resistance along with temperature sensitivity when chromosomal DNA extracted from this *ts* mutant was transformed into DB403. Just as we have expected, about 60% of the chloramphenicol-resistant transformants were found to be *ts* as well. Sequencing of the intact *sigA* gene in some of the *ts* transformants revealed that they all had the same *sigA* DNA sequence as that found in DB1005. Further evidence indicated the *ts* property of the mutant *sigA* allele was borne out by gene conversion experiments. In these experiments, a pYJ12 plasmid (see Materials and Methods) was constructed and transformed into DB1005. About 8% of the transformants were found to be able to grow on the 2× SG (24) plates at 55°C, which was about the same frequency as that observed previously in gene conversion studies (4, 21, 22). This result indicates that the substitution of the wild-type *sigA* gene for the mutant one on the chromosome of DB1005 would rescue the *ts* phenotype of DB1005. From the above-mentioned data, it is clear that the σ^A protein encoded by the intact *sigA* allele of DB1005 is *ts*.

Effects of temperature upshift on growth and synthesis of total RNA and total protein in DB2 and in DB1005. We have studied the effects of temperature upshift on the growth of DB2 and DB1005. Similar growth rate and growth potential were observed for DB2 and DB1005 in 2× SG liquid medium at 37°C. However, the growth of DB1005 stopped about 1 h after the upshift of temperature from 37 to 49°C, while DB2 remained multiplying vigorously (8). The growth properties of DB2 and DB1005 in a poorer medium (glucose minimal medium) were also studied. Our results showed that the growth rate and growth potential of DB1005 were lower than those of DB2 at 37°C (Fig. 1). Upshift or downshift of temperature exerted no significant effect on the growth of DB2 (Fig. 1A), whereas it had a drastic impact on DB1005. The growth of DB1005 stopped within 30 min after the change of temperature from 37 to 49°C, but it resumed after being shifted back to 37°C (Fig. 1B). The difference in the growth of DB1005 between these two media suggested that DB1005

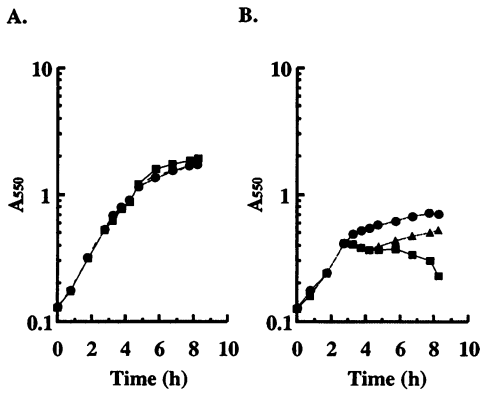

FIG. 1. Effects of temperature upshift and downshift on growth of *B. subtilis*. Shown are growth curves of DB2 (A) and DB1005 (B) in glucose minimal medium. The two strains of *B. subtilis* were both grown at 37°C to an optical density (A_{550}) of 0.4 before being aliquoted. One portion of the culture remained incubated at 37°C; the other two were transferred to 49°C. One of the portions transferred to 49°C was shifted back to 37°C 1.5 h later. ●, growth of *B. subtilis* at 37°C throughout the whole experiment; ■, cultures shifted from 37 to 49°C and then kept at 49°C; ▲, cultures shifted back to 37°C after 1.5 h of incubation at 49°C. Note that the growth curves of DB2 at 37°C and DB2 shifted back from 49 to 37°C are overlapping in panel A.

demanded more nutrition for normal growth. This notion paralleled the observation that DB1005 had a higher growth potential and was much less sensitive to heat stress when cultivated with a superrich medium (5). The nutritional demand was probably attributed to the lower transcription activity of the *ts* σ^A protein of DB1005. In addition, the resumption of growth of DB1005 after downshift of temperature and the constant viable counts of DB1005 at 49°C (5) suggested that most of the DB1005 cells survived the heat shock.

In order to understand the temperature sensitivity of DB1005, we investigated the total RNA and the total protein synthesis rates of DB2 and DB1005 under heat shock conditions (Fig. 2). Data from repeated experiments showed that the synthesis rate of total RNA in DB1005 declined steadily after being transferred from 37 to 49°C until min 6, and then it

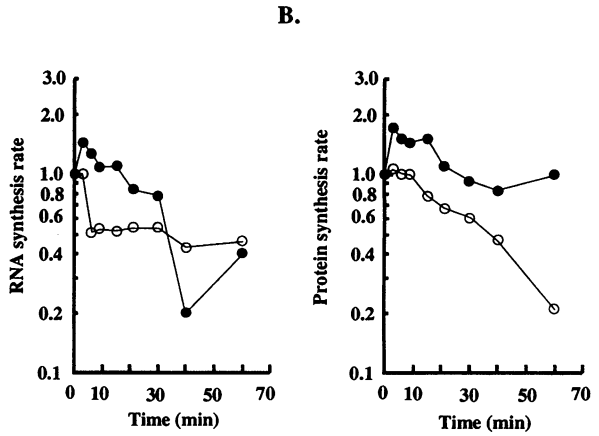

FIG. 2. Effects of temperature upshift on rates of total RNA (A) and total protein synthesis (B) in DB2 (●) and DB1005 (○). *B. subtilis* cells were pulse-labeled at the time points indicated in Materials and Methods. The rates of synthesis of total RNA and total protein were normalized to the rates before shifting from 37 to 49°C.

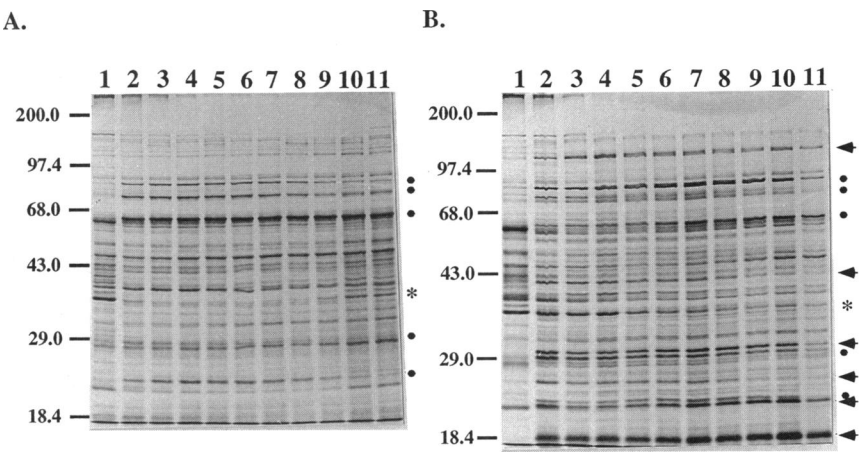

FIG. 3. Difference in the protein patterns between *B. subtilis* DB2 (A) and DB1005 (B) under heat stress. The autoradiograms were prepared as described in Materials and Methods. The running gels for SDS-PAGE were composed of 8% polyacrylamide. Lanes: 1, samples before heat shock; 2 through 11, samples pulse-labeled at 3, 6, 9, 12, 15, 18, 21, 30, 40, and 60 min, respectively, after heat shock. ●, proteins induced in both DB2 and DB1005; ◄—, proteins induced only in DB1005; *, protein repressed in both DB2 and DB1005. Molecular mass markers (in kilodaltons) are shown at the left of each panel.

leveled off (Fig. 2A). The synthesis rate of total protein also decreased in DB1005 under the same conditions (Fig. 2B). On the contrary, the synthesis of total RNA and total protein in DB2 shot to the highest rates and then dropped gradually, spanning a period of 21 min after being transferred to 49°C (Fig. 2). The differences in the patterns of total RNA and total protein synthesis rates between DB2 and DB1005 reflected that DB1005 responded abnormally to heat stress.

Protein patterns of DB2 and DB1005 under heat stress. The inability of DB1005 to grow at 49°C, we thought, was most probably due to its inability in initiating a normal heat shock response. To explore this possibility, the protein patterns of DB2 and DB1005 under heat stress were examined. Cell samples from both strains were pulse-labeled with [^{35}S]methionine for 10 min at designated times after the upshift of temperature from 37 to 49°C. Differences in the protein patterns were analyzed after SDS-PAGE and autoradiography. Proteins induced in both DB2 and DB1005 (Fig. 3) include the 80-, 74-, 64-, 29-, and 22-kDa proteins. Interestingly, the induction level was much lower in DB1005 than in DB2. On the basis of the molecular masses, the 74- and 64-kDa proteins were supposed to be DnaK and GroEL of *B. subtilis*, respectively. Proteins which were only abruptly induced in DB1005 are shown by arrows (Fig. 3B). The apparent molecular masses of these proteins were about 120, 42, 30, 25, 21, and 18 kDa. Furthermore, repression of certain proteins was also evident in DB2 and DB1005 under heat stress. One protein belonging to this class had a molecular mass of about 36 kDa (Fig. 3). In order to understand more about the response of DB1005, some of the above-mentioned proteins were N terminally sequenced, and the resultant amino acid sequences were aligned with those in the data library. By doing so, the 36-kDa (repressed rapidly in DB2 but slowly in DB1005), the 21-kDa (induced abruptly in DB1005 but not in DB2) and the 18-kDa (induced abruptly in DB1005 but not in DB2) proteins were confirmed to be the flagellin (31), alkyl hydroperoxide reductase (41), and glucose starvation-inducible protein B (32) of *B. subtilis*, respectively. Since the alkyl hydroperoxide reductase and the glucose starvation-inducible protein B were not in the category of heat shock proteins, we suggest that other cellular events, besides heat shock, are induced in the *ts sigA* mutant under heat stress.

Identification and expression of GroEL protein in DB2 and DB1005. The proteins which attracted most of our attention were induced abruptly in DB2 but slightly in DB1005. One protein belonging to this class and having a molecular mass of about 64 kDa was supposed to be GroEL of *B. subtilis*. To prove this point, we amplified the *groEL* gene from the chromosome of *B. subtilis* and cloned it into a pET-3 plasmid to overexpress the GroEL protein. The overexpressed protein was then gel purified and injected into rabbits for antibody production (see Materials and Methods). Immunodetection of the GroEL protein after 2-D gel electrophoresis is shown in Fig. 4. From the comparison of the heat shock protein patterns of these two 2-D gels, we found that there was a big difference in the amount of a protein with a molecular mass of about 64 kDa and a pI value of about 4.7. This protein was confirmed to be the GroEL of *B. subtilis* immunologically (Fig. 4). It was also clear that only a limited amount of this protein was synthesized in DB1005 compared with that of DB2.

A more-detailed comparison of the expression of GroEL protein in both DB2 and DB1005 during heat shock is shown in Fig. 5. Radioactivity of the GroEL protein in each lane of Fig. 3 was counted by a radioactivity image reader and plotted against the sampling time. It was found that the expression of GroEL protein in DB2 peaked at about 15 min after heat shock; however, maximal expression of this protein in DB1005 took 25 more min. In addition, threefold more GroEL protein was induced in DB2 than in DB1005, as judged by the incorporated radioactivities (Fig. 5A). This finding paralleled the observation of a rapid accumulation of GroEL protein in DB2 but not in DB1005 (Fig. 5B). In this investigation, equal volumes of lysate from DB2 and DB1005 were run on polyacrylamide gels and analyzed immunologically with a mixture of GroEL and σ^A antibodies (both antibodies have been shown to be reactive only with their respective target antigens). σ^A protein detected in this experiment served as a reference for monitoring the accumulation of GroEL protein in both *B. subtilis* strains. We found that the GroEL protein in DB2 started to accumulate 5 min after heat shock, but no such phenomenon was observed for σ^A in the same time period (Fig. 5B). On the contrary, there was a transient decrease in the amount of GroEL protein and a continuous decreasing

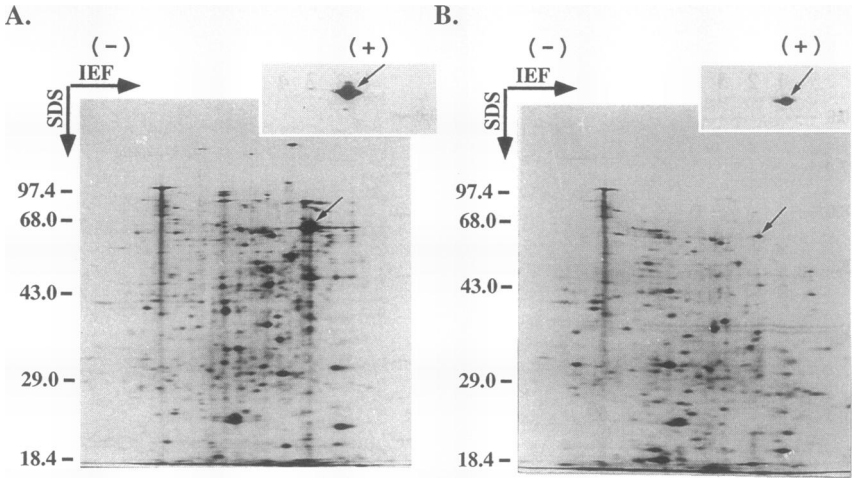

FIG. 4. Identification of the GroEL protein in DB2 (A) and DB1005 (B) by 2-D electrophoresis and immunoblotting. Methods for the preparation of the first-dimension gel were described in Materials and Methods. SDS-PAGE (with 8% polyacrylamide as a running gel) was used for the second dimension of analysis. The spots shown in insets along the upper right edges of each autoradiogram and indicated by arrows are the GroEL proteins detected. Numbers on the left margin of each figure are the apparent molecular masses (in kilodaltons) of marker proteins purchased from Bethesda Research Laboratories.

trend in the amount of σ^A protein in DB1005 under the same conditions as for DB2 (Fig. 5B).

Expression of *groEL* gene in vivo in both DB2 and DB1005 at the level of transcription. From the data of the previous section, it can be certain that the synthesis of GroEL protein is not significantly induced in DB1005 under heat shock conditions; however, it remains unclear whether expression of this gene is controlled at the transcription level, as is the case for *E. coli*. To unravel this mystery, we compared the amounts of *groEL* mRNA synthesized in vivo before and after heat induction in both DB2 and DB1005 by Northern blot analysis. In the analyses, three mRNA bands corresponding to the sizes of 2.3, 1.8, and 1.5 kb were observed in RNA samples isolated from cells cultivated in glucose minimal medium and Luria-Bertani medium (Fig. 6). On the basis of the size of the *groESL* operon, the 2.3-kb mRNA was supposed to contain message of the whole operon, whereas the 1.8- and 1.5-kb mRNAs were the premature or processed products of the 2.3-kb mRNA (39). Since the 1.8-kb mRNA was one of the predominant transcripts, we believed that certain sites on the *groESL* mRNA must be available for quick and easy processing. More importantly, there was a dramatic increase (about 15-fold) of the *groEL* mRNA in DB2 3 min after temperature upshift, but there was no such induction in DB1005 (Fig. 6). The accumulation of *groEL* mRNA in DB1005 was very slow after induction (Fig. 6B). This phenomenon suggests that the transcription of *groEL* gene in *B. subtilis* during heat shock is greatly affected by the replacement of the wild-type *sigA* with the *ts* one. In other words, a functional σ^A is required for the expression of the *groEL* gene under heat stress.

In vitro transcription of the putative σ^A-type promoter from the *groESL* operon by reconstituted RNA polymerase holoenzyme containing σ^A. Besides in vivo studies of expression of the *B. subtilis groEL* gene, an in vitro transcription assay was also performed to investigate the relation between σ^A and the putative σ^A-type promoter from the *groESL* operon (25, 39). In order to determine whether the putative σ^A-type promoter from the *groESL* operon was transcribed by σ^A, two additional control promoters were employed for in vitro transcription assays. One of them was the G3aG3b tandem promoter (σ^A type) from ϕ29 phage (11); this promoter was used as a positive control. The other was the P3 promoter (σ^H type) from the *B. subtilis sigA* operon, which was used as a negative control (3). Results of in vitro transcription of *groESL*, G3aG3b, and P3 promoters at both 37 and 49°C are shown in Fig. 7. As expected, no transcript from the P3 promoter was observed when it was transcribed by resconstituted RNA polymerase holoenzyme containing σ^A at both 37 and 49°C (Fig. 7, lane 3). However, transcripts of expected sizes (142 bases from the G3b promoter and 363 bases from the *groESL* promoter) were observed when G3aG3b and *groESL* promoters were transcribed by the same reconstituted σ^A RNA polymerase (Fig. 7, lanes 1 and 2). These results clearly indicate that the putative σ^A-type promoter from the *B. subtilis groESL* operon is at least specifically transcribed by σ^A at both 37 and 49°C in vitro.

Responses of DB2 and DB1005 to ethanol. Aside from heat stress, other forms of environmental stress can also stimulate the synthesis of heat shock proteins (1, 13, 23, 36). In view of the fact that changing the hydrophobicity of the hydrophobic face in the α-helix of region 2.4 of the σ^A protein yields the *ts sigA* mutant, we wondered whether the function of σ^A factor could also be affected by the organic solvent ethanol, which is less polar than H_2O. The possibility was examined by studying the ethanol responses of DB1005 and DB2. Cell samples from both strains of *B. subtilis* were treated with 4% ethanol and pulse-labeled with [^{35}S]methionine for 10 min. The samples were analyzed after SDS-PAGE and autoradiography. Similar sets of proteins, as indicated in Fig. 8, were induced within about 3 min in DB2 and DB1005 after ethanol shock. These proteins were mostly smaller than 32 kDa. However, the synthesis of a 27- and a 33-kDa protein increased significantly in DB1005 but not in DB2 about 15 min after the treatment of 4% ethanol. These results suggested that the *ts* σ^A mutant did not respond too differently from the wild type to ethanol stress, except after a relatively long exposure. Since the responses of heat shock and ethanol treatment differed significantly (Fig. 3A and 8A), we suggested that they were not regulated in the same way in *B. subtilis*.

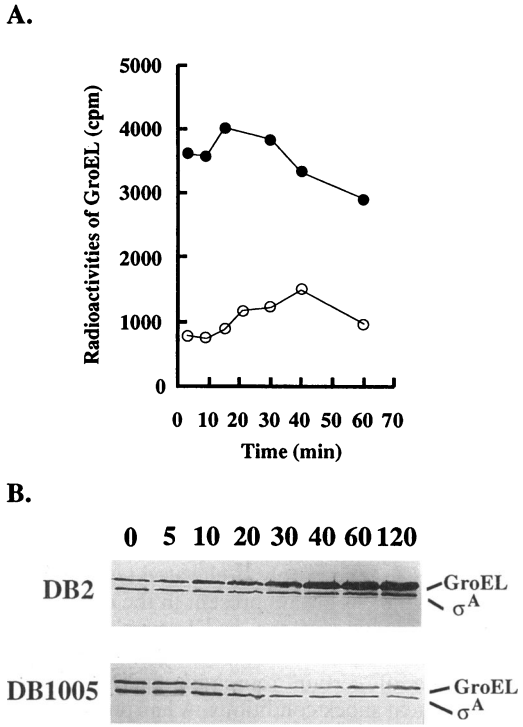

FIG. 5. Expression of the GroEL protein in DB2 and DB1005 under heat stress. (A) Synthesis of the GroEL protein. The radioactivity of each band corresponding to the GroEL protein in Fig. 3 was read by a radioactivity image reader and plotted against the time for pulse-labeling with [^{35}S]methionine. ●, Radioactivities incorporated in the GroEL protein of DB2; ○, radioactivities incorporated in the GroEL protein of DB1005. (B) Accumulation of GroEL and σ^A proteins in DB2 and DB1005 during heat shock. *B. subtilis* cultures (1.5 ml) heat shocked at an optical density (A_{550}) of 0.4 were pelleted, resuspended in 50 μl of 0.5× SET buffer (20% sucrose, 50 mM Tris-HCl [pH 7.6], 50 mM EDTA, 2 mM phenylmethylsulfonyl fluoride), and disrupted in 50 μl of 2× sample application buffer (5) by being heated at 90°C for 3 min and vortexed for 5 min. Equal volumes of the samples were then used for immunological analyses of the GroEL and σ^A proteins. Numbers above each lane indicate the time (in minutes) at which samples from *B. subtilis* DB2 and DB1005 were taken after the cultures were shifted from 37 to 49°C.

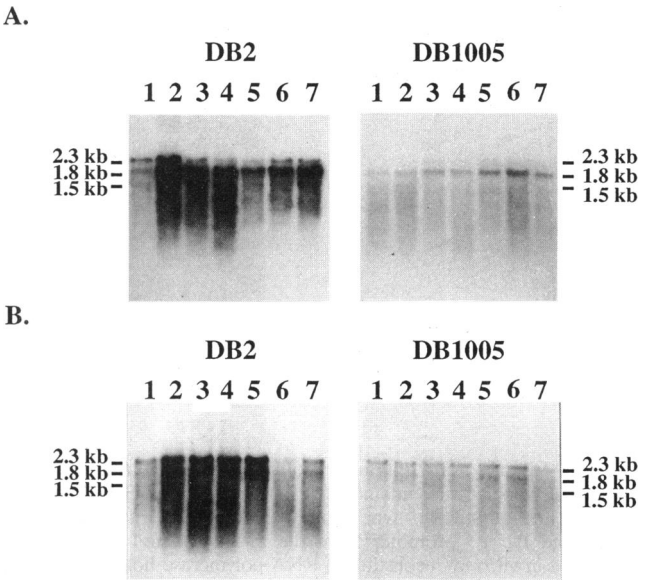

FIG. 6. Northern blot analyses of *groEL* mRNA. Shown are hybridizations of transcripts from *B. subtilis* DB2 and DB1005 cultivated in glucose minimal medium (A) and Luria-Bertani medium (B). Probe used for the analysis is a 1,062-bp *Hin*dIII-*Pst*I DNA fragment from the *groEL* gene (20). The strain of *B. subtilis* from which RNA was isolated is noted on the top of each panel. About 20 μg of the isolated RNA sample was loaded on each lane. Lanes: 1, hybridization of RNA isolated before heat shock; 2 through 7, hybridization of RNA isolated at 3, 9, 15, 21, 30, and 40 min after being shifted from 37 to 49°C. Numbers on the left margin of each figure are the sizes of transcripts.

DISCUSSION

We have confirmed that the intact *sigA* gene in DB1005 is a *ts* allele and this allele is transferrable among *B. subtilis* strains. This property is useful for further study of the σ^A-related gene regulation in *B. subtilis*.

We have also characterized the nature of temperature sensitivity of DB1005. This mutant responds abnormally to heat stress (Fig. 3 and 4). Reasons for the unexpected protein pattern of DB1005 under heat stress were very complicated, but it was unlikely to be caused by the death of DB1005 at elevated temperatures, because its viable count remained fairly constant during heat shock (5). It was more likely that other cellular events were triggered in DB1005 during heat shock. This notion was supported by an abrupt induction of two other stress proteins, i.e., alkyl hydroperoxide reductase (41) and glucose starvation-inducible protein B (32) (Fig. 3) in DB1005 during heat shock. The induction of these two proteins indicates that DB1005 also suffers from oxidative stress and glucose starvation when subjected to heat shock. It also suggests that the *ts sigA* allele has a pleiotropic effect on gene expression of DB1005 under heat stress. The mechanisms which result in the pleiotropic effect and the relation of this effect to the *ts* σ^A are currently unknown. However, it is possible that induction of repressors or other control mechanisms is also affected by the *ts* σ^A protein or ancillary control factors under heat stress.

Although the mechanism for living cells to adapt themselves to higher temperatures is not fully understood, the functions of certain heat shock proteins are quite clear (9, 12, 28, 29). The GroEL protein in our focus is a chaperonin required for proper folding and prevention of denaturation of cellular proteins; thus, limited expression of the *groEL* gene at elevated temperatures (Fig. 5 and 6) might result in the easy degradation of cellular proteins and the cessation of growth of cells. The lack of essential chaperonins as well as its suffering from oxidative stress and glucose starvation, we thought, might be directly or indirectly responsible for the loss of growth potential of DB1005 at elevated temperatures.

Besides the above findings, we found either that σ^A was uninducible or that its induction was undetectable during heat shock (Fig. 5B). This phenomenon is quite different from that reported for *E. coli* σ^{70}, which increases about three- to fivefold after heat shock (15, 45). Thus, the heat shock responses of *B. subtilis* and *E. coli* are regulated differently, and *B. subtilis* might not have an extra σ factor for controlling the heat shock response as *E. coli* does; the latter possesses σ^{32} besides σ^{70}.

The inability of the *ts sigA* mutant to express, in vivo, the *groEL* gene at both transcription and translation levels under heat stress (Fig. 5 and 6) as well as the specificity of σ^A in transcribing the putative σ^A-type promoter from the *groESL* operon in vitro (Fig. 7) manifests that the *groEL* gene is

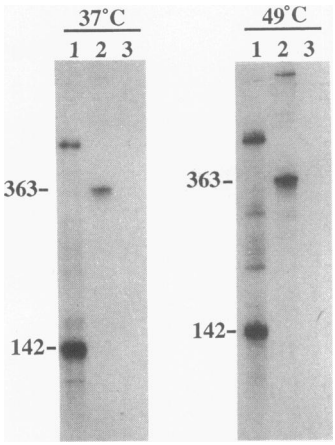

FIG. 7. In vitro transcription of promoters with reconstituted RNA polymerase holoenzyme containing σ^A. The ϕ29 phage G3aG3b tandem promoter, the putative σ^A-type promoter from the *B. subtilis groESL* operon and the P3 promoter from the *B. subtilis sigA* operon were transcribed in vitro by reconstituted RNA polymerase holoenzyme containing σ^A at both 37 and 49°C. The KCl concentration of the reaction mixtures for the above three promoters was 150, 100, and 100 mM, respectively. Lane 1, transcripts from the G3aG3b promoter; lane 2, transcripts from the putative σ^A-type promoter of *groESL* operon; lane 3, transcripts from the P3 promoter. Numbers on the left margin of each panel indicate the sizes (in bases) of transcripts. Note that transcripts with sizes above 363 bases are not products from specific promoters.

regulated at least in part by σ^A at the level of transcription under heat shock conditions. This argument can be further strengthened by several lines of evidence. First, the *groESL* operon has a putative σ^A-type promoter, and no other DNA sequences recognizable by other known σ factors have been found (25, 47). Second, if an analog of *E. coli* σ^{32} was involved in regulating the heat shock response of *B. subtilis*, we would expect to see a similar heat shock response between DB1005 and the *ts rpoD* mutants of *E. coli* under heat stress (16, 35). However, the response of DB1005 to heat stress is quite different from that of the *E. coli rpoD800*. Despite a rapid degradation of the *ts* σ^{70} in *lon*$^+$ cells, the *rpoD800* mutant responds to heat shock in greater magnitude and longer duration (16, 17). In contrast, no such response in DB1005 was observed. Third, the heat shock response of DB1005 is quite similar to that of the *E. coli htpR* nonsense mutants in that the induction of *groEL* gene is very slow and only a small amount of the GroEL protein is synthesized in both organisms upon transfer to elevated temperatures (48, 49). All these results point to the similar regulatory roles played by σ^A and σ^{32} in controlling the *groEL* genes. In other words, σ^A is intimately related to the expression of the *groEL* gene in *B. subtilis*.

The evidence we present here concerns only the expression of *groEL* and possibly also *dnaK*; hence, it is too early to suggest that σ^A controls a heat shock regulon of *B. subtilis* unless more data are available from the studies of the regulation of *B. subtilis* heat shock protein genes. In fact, it is still possible that certain heat shock genes of *B. subtilis* are directed by other σ factors. It is also of interest to know why the activation of *groEL* gene is only at elevated temperatures (Fig. 5 and 6), although σ^A is always present in the cells. The reason could be that induction of the *groEL* gene requires certain ancillary factors which are available only under heat stress or that elimination of certain repressing factors can only be possible under heat shock conditions. A hairpin loop located 5′ to the *groESL* and *dnaK* operons in conjunction with a repressor has been proposed recently to be responsible for the activation of these operons, but how this system works is still not understood (40).

Our data also showed that the ethanol response of DB1005 was not too different from that of DB2, except after a relatively long exposure. The reason for this difference remains unclear; however, it is possible that ethanol acts differently on the *ts* σ^A in transcribing different categories of genes. More specifically, ethanol has no significant effect on the *ts* σ^A in transcribing the category of genes which are induced early, whereas it affects

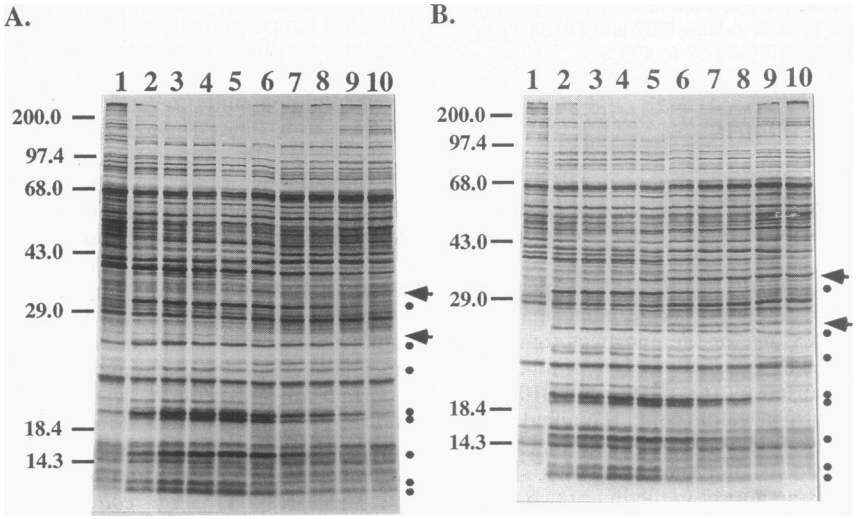

FIG. 8. Ethanol induction of heat shock proteins. *B. subtilis* DB2 (A) and DB1005 (B) were grown in glucose minimal medium at 37°C to an optical density (A_{550}) of 0.4. Ethanol treatment (4% [vol/vol]) and pulse-labeling were then carried out. Lanes: 1, before ethanol treatment; 2 through 10, samples pulse-labeled at 3, 6, 9, 15, 21, 30, 40, 60, and 120 min after the addition of ethanol, respectively. Equal amounts of protein from each cell sample were fractionated by SDS-PAGE with a 12.5% polyacrylamide gel. ●, proteins induced in both strains; ◄–, proteins induced later in DB1005 but not in DB2. Numbers on the left margin of each figure are the apparent molecular masses (in kilodaltons) of proteins as determined by SDS-PAGE.

the transcription of another category of genes which are induced later. It would be interesting if the late induction is an indication of the response of the *ts* σ^A to the change of hydrophobicity in the environment after ethanol treatment.

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