The temperature sensitivity of *Bacillus subtilis* DB1005 is due to insufficient activity, rather than insufficient concentration, of the mutant σ^A factor

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The σ^A factor of *Bacillus subtilis* DB1005 contains two amino acid substitutions (I198A and I202A) in the promoter –10 binding region. It has been confirmed that this σ factor is responsible for the temperature sensitivity of *B. subtilis* DB1005. An investigation was conducted into how the mutant σ^A could cause temperature-sensitive (Ts) cell growth by analysing its structural stability, cellular concentration and transcriptional activity. The mutant σ^A was unstable even at the permissive temperature of 37 °C ($t_{1/2}$ 59 min), whereas the wild-type counterpart was fairly stable under the same conditions ($t_{1/2}$ > 600 min). However, neither wild-type σ^A nor mutant σ^A was stable at 49 °C ($t_{1/2}$ 34 min and 23 min, respectively). Analyses of the rates of σ^A synthesis revealed that *B. subtilis* DB1005 was able to compensate for unstable σ^A by elevating the level of σ^A at 37 °C but not at 49 °C. Moreover, overexpression of the mutant σ^A at 49 °C could not suppress the Ts phenotype of *B. subtilis* DB1005. This indicates that the temperature sensitivity of *B. subtilis* DB1005 is not due to insufficient σ^A concentration in the cell. The greater decline of an already reduced activity of the mutant σ^A at 49 °C suggests that the temperature sensitivity of *B. subtilis* DB1005 is instead the result of a very low activity of σ^A ; probably below a critical level necessary for cell growth.

Keywords: *Bacillus subtilis*, σ^A factor, α -helix, degradation rate, temperature sensitivity

INTRODUCTION

σ factors direct RNA polymerase to the promoter sites on DNA molecules and initiate the transcription of DNA (Burgess *et al.*, 1969). Sequence alignment of a large number of σ factors has revealed four conserved regions in this group of proteins (Helmann & Chamberlin, 1988; Lonetto *et al.*, 1992). Genetic studies revealed that region 4.2 and region 2.4 are involved in the recognition of promoter DNA (Daniels *et al.*, 1990; Gardella *et al.*, 1989; Kahn & Ditta, 1991; Siegele *et al.*, 1989; Tatti *et al.*, 1991; Zuber *et al.*, 1989). Region 4.2 at the C-terminus contains a helix–turn–helix motif which is thought to interact specifically with the –35 region of the promoter (Brennan & Matthews, 1989; Gardella *et al.*, 1989; Siegele *et al.*, 1989). Region 2.4 forms an amphiphilic α -helix; certain amino acid

residues on this helix are believed to participate in sequence-specific contacts with promoter –10 DNA (Daniels *et al.*, 1990; Siegele *et al.*, 1989; Tatti *et al.*, 1991; Waldburger *et al.*, 1990). Amino acids which confer altered –10 recognition properties are presumably on the hydrophilic face of the amphiphilic α -helix in *Escherichia coli* σ^{70} as well as in *Bacillus subtilis* σ^A , σ^E , σ^F and σ^H (Daniels *et al.*, 1990; Kenney *et al.*, 1989; Kenney & Moran, 1991; Siegele *et al.*, 1989; Tatti *et al.*, 1991; Zuber *et al.*, 1989). Thus, amino acids on the hydrophobic face of the promoter –10 binding helix must play different roles.

In order to clarify the roles of the conserved hydrophobic amino acids on the hydrophobic face of the promoter –10 binding helix of σ^A , we constructed several *B. subtilis* *sigA* mutants which contained amino acid substitutions in this region by using site-directed mutagenesis (Chang & Doi, 1993b). One of the mutants has two amino acid substitutions, I198A and I202A, on the

Abbreviation: Ts, temperature-sensitive.

Fig. 1. Amino acid substitutions in the α -helix of the promoter -10 binding region of *B. subtilis* σ^A . (a) The top two lines indicate the number and sequence of amino acids in the promoter -10 binding region of the wild-type σ^A . The bottom two lines indicate the sense strand DNA sequences of the wild-type *sigA* gene of *B. subtilis* DB2 and the mutant *sigA* gene of *B. subtilis* DB1005. The two substituted alanine codons in DB1005 *sigA* gene are boxed. (b) Helical wheel representation of the amphiphilic promoter -10 binding helix of DB1005 σ^A . The substituted alanine residues at positions 198 and 202 are circled. (c) Cylindrical plot of the amphiphilic α -helix of the promoter -10 binding region of σ^A . The substituted alanine residues at positions 198 and 202 are indicated by filled circles.

hydrophobic face (Fig. 1) and confers a temperature-sensitive (Ts) phenotype on *B. subtilis* DB1005. The growth of this mutant stops within 30 min after the temperature upshift (37 to 49 °C) and resumes after the downshift (49 to 37 °C). In addition, it suffers from starvation and oxidative stresses at the restrictive temperature (Chang & Doi, 1993b; Chang *et al.*, 1994). We are interested in determining the nature of the mutant σ^A causing the temperature sensitivity of DB1005.

It has been reported that the promoter -10 binding region is within the most hydrophobic domain of σ^A and is resistant to protease attack (Chang & Doi, 1990). Moreover, free σ^A is unable to bind promoter DNA unless it associates with RNA polymerase core enzyme and undergoes a conformational change (Chang & Doi, 1993a). The unexposed nature of the promoter -10 binding region suggests that the replacement of Ile-198 and Ile-202 with alanines would affect the interdigital interaction between amino acids in these two positions and those in other region(s) of σ^A both in free- and core-enzyme-associated states. Thus, the temperature sensitivity of *B. subtilis* DB1005 might be due to a certain change in σ^A structure, which in turn leads to degradation or altered function of the mutant σ^A , or a combination of both factors, at elevated temperatures.

We have analysed some properties of the mutant σ^A factor. Our data showed that *B. subtilis* DB1005 σ^A was unstable at both permissive and restrictive temperatures, while the wild-type σ^A was unstable only at the elevated temperature. We also showed that a higher mutant σ^A concentration was unable to suppress the Ts phenotype, supporting the idea that the temperature sensitivity of *B.*

subtilis DB1005 is not caused by insufficient σ^A concentration. The marked reduction of DB1005 σ^A activity at high temperature and its correlation with the temperature sensitivity of *B. subtilis* DB1005 suggests that it is below a critical level necessary for cell growth under heat stress.

METHODS

Enzymes and chemicals. Restriction enzymes, ligase and other DNA modification enzymes were purchased from BRL and used according to the recommendations of the manufacturer. Anti- σ^A and anti-GroEL antibodies were prepared as described previously (Chang & Doi, 1990; Chang *et al.*, 1994). Protein A-Sepharose CL-4B was purchased from Pharmacia. L-[35 S]methionine [1200 Ci mmol $^{-1}$ (44.4 TBq mmol $^{-1}$)] and [α - 32 P]dCTP [3000 Ci mmol $^{-1}$ (111 TBq mmol $^{-1}$)] were obtained from Amersham. Other reagents were products of Merck and Serva.

Bacterial strains. *B. subtilis* strains DB1005 [*trpC sigA* (Ts)], DB430 (*trpC npr apr epr bpf ispl*) (He *et al.*, 1991) and DB435 [*trpC npr apr epr bpf ispl sigA* (Ts)] are derivatives of DB2 (*B. subtilis* 168 *trpC*). To construct *B. subtilis* DB435, chromosomal DNA extracted from DB1005 was transformed into *B. subtilis* DB430. Transformants which had the same Ts phenotype and *sigA* DNA sequence as those of DB1005 were named DB435.

Construction of plasmids containing wild-type or mutant *sigA* genes expressible in *B. subtilis*. To construct the pCX2F plasmid harbouring the wild-type *sigA*, an *EcoRI* DNA fragment containing the P1P2 promoter from *B. subtilis* *sigA* operon was first cut out from the pUC19-43L plasmid and cloned into the promoter probing vector, pWP18 (Wang & Doi, 1987) to form the plasmid pCC1. This was then cleaved with *Sall* to remove the *aprE* gene, leaving the vector part of

the plasmid to self-ligate for the generation of the plasmid pCC2. Afterwards, a *Bgl*III DNA fragment containing the wild-type *sigA* gene (including the ribosome-binding sequence) was excised from the σ^A -overexpressing plasmid, pCD2 (Chang & Doi, 1990), and cloned into the *Bam*HI site of pCC2 to form pCX2F. This construction allowed the *sigA* gene on pCX2F to be controlled both by the transcriptional signal (P1P2 promoter) of the *sigA* operon and by the translational signal of the *sigA* gene (Wang & Doi, 1986). For some reason, the *sigA* gene of *B. subtilis* DB1005 was difficult to clone with conventional cloning strategies. To overcome this difficulty, the method of gene conversion (Chak *et al.*, 1982; Iglesias & Trautner, 1983) was adopted. In this approach, the pCX2F plasmid was introduced into *B. subtilis* DB1005 and then transformants with kanamycin (Km)-resistant and Ts phenotypes were screened. This type of transformant contained plasmid pCX2F5, in which the wild-type *sigA* allele has been replaced with the Ts *sigA* allele of *B. subtilis* DB1005. To obtain a *B. subtilis* DB1005 strain with a pure population of pCX2F5, plasmids in the aforementioned Ts transformants were extracted and reintroduced into *B. subtilis* DB1005. The secondary Ts transformants thus obtained were further streaked and purified; these cells contained the mutant *sigA* gene on the plasmid as determined by direct sequencing of the *sigA* DNA.

Degradation rate of σ^A and GroEL proteins. To determine the degradation rate of σ^A protein *in vivo*, each *B. subtilis* strain was grown in glucose minimal medium (GMM) (Spizizen, 1958) supplemented with 0.004% tryptophan at 37 °C to an OD₅₅₀ of 0.4 (measured with a Milton Roy Company Spectronic 20D spectrophotometer) and the culture was separated into two portions. One of the aliquots was further incubated at 37 °C; the other was transferred to 49 °C. Nine minutes later, the cultures were pulse-labelled with L-[³⁵S]methionine (1200 Ci mmol⁻¹) at a concentration of 20 µCi ml⁻¹ for 5 min and then chased with nonradioactive methionine at a concentration of 5 mM. Sampling of the labelled cultures was started 2 min later. At the designated time points, 0.5 ml of each culture was removed, pelleted by centrifugation at 4 °C for 10 min and then incubated at 37 °C for 10 min in 40 µl lysis buffer (50 mM glucose, 25 mM Tris/HCl pH 8.0, 0.1 mg lysozyme ml⁻¹, 0.2 mM PMSF). Afterwards, 20 µl detergent solution (1% (w/v) SDS, 1% (v/v) Triton X-100) was added to each sample to disrupt the cells. The sample was then heated to 90 °C for 3 min and insoluble cell debris in the samples was removed by centrifugation. To specifically precipitate σ^A and GroEL proteins in the supernatant simultaneously, a 50 µl aliquot of the sample was diluted with 450 µl of incubation buffer (1% Triton X-100, 0.1% SDS, 100 mM NaCl, 10 mM Tris/HCl pH 8.0) prior to the addition of 1 µl each of anti- σ^A and anti-GroEL antibodies. The mixture was incubated overnight at 4 °C with gentle shaking (100 r.p.m.) and 60 µl of swollen protein A-Sepharose CL-4B in incubation buffer was added to the sample mixture before shaking for another 2 h at 4 °C. The Sepharose resin was pelleted, washed once with incubation buffer (1 ml) and once with Tris/HCl-buffered saline solution (100 mM NaCl, 10 mM Tris/HCl pH 8.0). After being vacuum-dried, proteins absorbed by the resin were solubilized with 30 µl Laemmli sample buffer (Laemmli, 1970) for 10 min at 90 °C. Ten microlitres of solubilized protein solution of the sample was electrophoresed on SDS-polyacrylamide gel. The protein gels were stained with Coomassie blue, vacuum-dried and exposed to X-ray film. Radioactivities of the target protein bands were measured with an Ambis Radioactivity Image Reader.

Overexpression of the mutant σ^A protein. To overexpress *B. subtilis* DB1005 σ^A , the chromosomal DNA of the bacterium was extracted and used as template for amplification of the *sigA* DNA. Nucleotide sequences of the forward and reverse primers were 5'-CTGCAGAGATCTGAATTCGTTGCAA-GCTTTGG-3' and 5'-CTGCAGAGATCTGTATACGCTT-ACAATAGAAA-3', respectively. The amplified *sigA* gene contains its own ribosome-binding site as well as an *Eco*RI and a *Bgl*III site at the 5' ends of the primers. After digesting with *Eco*RI and *Bgl*III, the *sigA* DNA fragment was cloned into the *Eco*RI and *Bam*HI sites of the overexpression vector, pT7-5 (Tabor, 1990), to form pCT12. The *sigA* DNA sequence on pCT12 was examined and confirmed to be correct. Overexpression and purification of DB1005 σ^A protein from the culture of *E. coli* BL21 (DE3) were done as reported previously (Chang & Doi, 1990).

Measurement of σ^A activity *in vivo* at 37 °C. To measure the activities of wild-type and mutant σ^A factors at 37 °C, the *groE* promoter of *B. subtilis* (Li & Wang, 1992; Schmidt *et al.*, 1992) was first cloned into an integration vector, pCoiZA (Qi, 1990; Qi *et al.*, 1991). The resultant plasmid was then separately incorporated into the *aprE* gene of *B. subtilis* DB2 and DB1005. Sequences for the forward and reverse primers for the synthesis of *groE* promoter DNA were 5'-AGATCTGCAGATCGCGATTAATGCGG-3' and 5'-GTTAGGATCCTCA-ATAGTATGAATATGT-3', respectively. The *groE* promoter DNA contained a *Pst*I and a *Bam*HI site which were used for cloning. The *groE* promoter DNA on the integration plasmid was sequenced and proved to be correct. β -galactosidase activities in both *B. subtilis* DB2 and DB1005 were measured by growing cells at 37 °C in 2×SG medium (Leighton & Doi, 1971) containing erythromycin (1 µg ml⁻¹), followed by harvesting at various growth stages and assaying activity by the method of Miller (1972).

RNA extraction and slot blot analysis of *lacZ* message. Methods for RNA extraction and quantification were as previously reported (Chang *et al.*, 1994). The RNA samples were transferred to a piece of Hybond-N membrane (Amersham) via a vacuum blotter; methods and conditions for hybridization analysis of the RNA were as those reported for Northern blot analysis (Chang *et al.*, 1994). The probe DNA was a *lacZ* DNA fragment flanked by *Xba*I and *Sst*I (about 2.0 kb in length), which was obtained by cutting the pCoiZA plasmid with *Xba*I and *Sst*I restriction enzymes. This DNA fragment was labelled with [α -³²P]dCTP using the random priming system purchased from Boehringer Mannheim.

Effect of temperature on transcription of DB1005 σ^A *in vitro*.

Core enzyme and σ^A were prepared as described elsewhere (Davison *et al.*, 1979; Fukuda & Doi, 1977; Halling *et al.*, 1977). Procedures for RNA polymerase holoenzyme reconstitution and *in vitro* transcription assay were modified according to a previously published method (Chang & Doi, 1990). In the present study, the RNA polymerase holoenzyme was reconstituted on ice for 10 min by mixing 3 µl core enzyme (3 µg) with 3 µl purified σ^A (1.5 µg). Afterwards, 3 µl *groE* promoter DNA (0.15 µg) was added and the mixture was further incubated at 37 °C for 5 min. Following this, the RNA polymerase and promoter DNA mixture was shifted to the designated temperature (32, 37, 42, 47 or 49 °C) and 50 µl reaction cocktail prewarmed at that temperature was then added to start the transcription reaction. The final concentration of each component in the reaction mixture was 40 mM Tris/HCl pH 7.9; 10 mM MgCl₂; 150 mM KCl; 20 mM DTT; 0.2 mM each of UTP, CTP, GTP and ATP; 5% glycerol; 3 µCi [α -³²P]ATP. The transcription reaction was

carried out at the designated temperature for 10 min and then an equal volume of stop solution (95 % formamide, 20 mM EDTA, 0.05 % bromophenol blue, 0.05 % xylene cyanole FF) was added. The sample of each transcription reaction was directly applied to an 8 M urea denaturing gel for electrophoretic separation of RNA. The *groE* promoter DNA template was 651 bp in length and would generate an RNA transcript of 363 bases from the transcription initiation site (Chang *et al.*, 1994). The G3b promoter DNA of *B. subtilis* ϕ 29 phage (Davison *et al.*, 1980) was synthesized by PCR (forward and reverse primers were 5'-AGAGAACGTAGACAACAAC-3' and 5'-CCTGAACCTCTCCGTCCAC-3', respectively). This promoter DNA template was 235 bp in length and would generate a transcript of 142 bp. Radioactivity of the target transcript in each sample was measured with an Ambis Radioactivity Image Reader.

RESULTS

The half-lives of wild-type and mutant σ^A factors

It is generally believed that hydrophobic interactions in the core region of some proteins are essential to their integrity. The promoter – 10 binding region is within the most hydrophobic part of σ^A protein (Chang & Doi, 1990), thus the two amino acid substitutions (I198A and I202A) (Fig. 1) which change the hydrophobicity of this region could affect the structural stability and degradability of σ^A , especially at elevated temperatures. To verify this, the degradation rates of both wild-type and mutant σ^A proteins were analysed *in vivo*. In the experiments, the total cellular proteins of *B. subtilis* DB2 and DB1005 were pulse-labelled at designated time points at both 37 and 49 °C followed by immunoprecipitation with anti- σ^A and anti-GroEL. Radioactivities of the protein bands corresponding to σ^A and GroEL were read with a radioactivity image reader after SDS-PAGE. Percentages of σ^A and GroEL remaining in the cells at each time point were calculated. The rate of GroEL degradation was used as an internal standard to monitor the performance of our experiments. The degradation rates of wild-type and mutant σ^A , as well as the degradation rates of GroEL in both DB2 and DB1005 at 37 °C are depicted in Fig. 2(a, c). Their half-lives, calculated according to the regression lines in Fig. 2(a, c), are shown in Table 1. We found that the degradation rates of GroEL at 37 °C in both DB2 ($t_{1/2}$ 158 min) and DB1005 ($t_{1/2}$ 152 min) were similar; however, the rates of σ^A degradation in these two strains were different. The relatively constant amount of labelled σ^A remaining in DB2 at 37 °C indicated that the wild-type σ^A was fairly stable ($t_{1/2}$ > 600 min) at this temperature. In contrast, the rapid decrease in the amount of labelled σ^A in DB1005 indicates that the mutant σ^A is relatively unstable ($t_{1/2}$ 59 min), even at the permissive temperature.

The degradation rates of σ^A and GroEL at 49 °C are shown in Fig. 2(b, d). The half-lives of GroEL in DB2 and DB1005, calculated according to the regression lines in Fig. 2(b, d) were about 42 and 57 min, respectively (Table 1). Moreover, two phases of σ^A degradation were observed in these two strains (Fig. 2b). The first phase was detected within the initial 40 min after the upshift of

temperature to 49 °C. In this phase, the degradation of wild-type σ^A in *B. subtilis* DB2 accelerated, with a half-life of about 34 min (Table 1). A similar magnitude of half-life was observed for the mutant σ^A in DB1005 ($t_{1/2}$ 23 min). These results indicate that both wild-type and mutant σ^A become unstable and more susceptible to protease after temperature elevation. The second phase of σ^A degradation was manifested after a prolonged incubation of the labelled cells at 49 °C. The percentages of labelled σ^A in the two strains remained fairly constant in this phase; however, a higher percentage of σ^A persisted in DB2 as compared with that in DB1005 (Fig. 2b). The reason for the occurrence of the second phase of σ^A degradation is unclear. One possibility is that a certain proportion of the labelled σ^A proteins, both wild-type and mutant, fail to dissociate from the RNA polymerase holoenzyme and are therefore protected from being degraded at the elevated temperature. Alternatively, it could represent the stabilizing effect of chaperonins induced under heat shock.

The lower degradation half-life of DB1005 σ^A could be due to the presence of intracellular proteases in the cells. The participation of the major intracellular protease, IspI, (Kiode *et al.*, 1986) in the degradation of σ^A in *B. subtilis* DB1005 was investigated by comparing the half-lives of the mutant σ^A in both *B. subtilis* DB1005 (*isp*⁺) and DB435 (*isp*) containing the same Ts *sigA* allele. Our data showed that σ^A in *B. subtilis* DB435 was degraded to a lesser extent than in DB1005 (Fig. 2a, b). The half-lives of σ^A in DB435 were slightly longer than those for *B. subtilis* DB1005 at both 37 and 49 °C (80 vs 59 min and 25 vs 23 min at 37 and 49 °C, respectively) (Table 1). Since *B. subtilis* DB435 and DB1005 showed a similar Ts phenotype, it appeared that the partially improved half-life of the mutant σ^A in the *isp* background could not alleviate the Ts phenotype.

B. subtilis DB1005 compensates for σ^A instability by increasing σ^A synthesis at 37 °C but not at 49 °C

σ^A in *B. subtilis* DB1005 was unstable at both 37 and 49 °C (Fig. 2, Table 1); however, it was only at 49 °C that the detrimental effect on growth was manifested (Chang & Doi, 1993b; Chang *et al.*, 1994). The difference is intriguing. A plausible answer to this puzzle based on our previous observations of a higher σ^A concentration in DB1005 relative to that in DB2 at 37 °C (Chang *et al.*, 1994) is that DB1005 may compensate for unstable σ^A by synthesizing and maintaining a sufficient concentration of σ^A in the cells at 37 °C but not at 49 °C. In other words, the temperature sensitivity of *B. subtilis* DB1005 at 49 °C was probably caused by its inability to sustain a sufficient concentration of σ^A in the cells. To verify this, we compared the residual amounts of the pulse-labelled σ^A and the rates of σ^A synthesis in both DB2 and DB1005 at different intervals since the cellular concentration of a protein is affected by its rates of degradation and synthesis at a specific time point. At 37 °C, the labelled σ^A remaining in DB1005 was higher relative to that in DB2 at each time point tested (Table

Fig. 2. Degradation of σ^A and GroEL proteins. (a, b) Regression lines of the means of triplicate determinations of σ^A remaining in the cells at (a) 37 °C and (b) 49 °C. (c, d) Regression lines of the means of triplicate determinations of GroEL remaining in the cells at (c) 37 °C and (d) 49 °C. Standard deviations were 10% of the means. Each data point represents the ratio of the radioactivity of σ^A or GroEL to that at time zero. Measurements for each regression line were repeated at least twice and were reproducible. ●, DB2; ▲, DB1005; △, DB435.

Table 1. Degradation of SigA and GroEL in *Bacillus subtilis*

Strain	Relevant genotype	Half-life (min)*			
		37 °C		49 °C	
		SigA	GroEL	SigA	GroEL
DB2	<i>sigA</i> (wild-type)	> 600	158	34	42
DB1005	<i>sigA</i> (Ts)	59	152	23	57
DB435	<i>sigA</i> (Ts) <i>npr apr epr bpf ispl</i>	80	171	25	74

*Determined according to the regression lines in Fig. 2.

Table 2. Relative amounts of SigA remaining and synthesized in *Bacillus subtilis*

Each value is the ratio of the radioactivity of labelled SigA in DB1005 to that in DB2 at the designated time point.

Time (min)	SigA remaining* (DB1005/DB2)		SigA synthesized† (DB1005/DB2)	
	37 °C	49 °C	37 °C	49 °C
0	2.46	0.92	1.85 (1.00)	1.06
10	2.01	0.83	1.54 (0.96)	—
20	1.95	0.68	1.89 (0.94)	—
30	1.60	0.58	1.60 (0.92)	—
40	1.46	0.51	2.02 (0.89)	—
50	1.35	0.48	2.46 (0.87)	—
60	1.18	0.50	1.92 (0.88)	—
80	0.97	0.44	2.66 (0.83)	—

—, Not measured.

*Relative amount of SigA remaining in the cell after chasing. Each value was determined on the basis of the data shown in Fig. 2.

†The cells were pulse-labelled at the designated time points for 5 min before sampling and determination of the amount of labelled SigA protein. Numbers in parentheses indicate the ratios of the OD₅₅₀ of DB1005 to that of DB2.

2). The ratio of labelled σ^A remaining in DB1005 and DB2 was about 2.46 to 0.97 within the 80 min interval and the relative amount of σ^A synthesized in DB1005 was at least 1.5-fold higher than that in DB2 at the same intervals at 37 °C. The higher residual amount of σ^A , along with the higher rate of σ^A synthesis in DB1005 implied that DB1005 was able to compensate for σ^A instability by synthesizing and maintaining a sufficient concentration of this factor in the cells at 37 °C.

The relative amount of labelled σ^A remaining in DB1005 and DB2 at 49 °C is also shown in Table 2. In contrast to the results observed at 37 °C, the amount of labelled σ^A remaining in DB1005 was less than that found in DB2. The ratio of σ^A remaining in DB1005 and in DB2 ranged from about 0.92:1 to 0.44:1 within the 80 min interval (Table 2). The ratio of the amount of σ^A synthesized in DB1005 and in DB2 was about 1.06 to 1 at 9 min after the upshift of temperature to 49 °C, which was much lower than the ratio of 1.85 to 1 obtained at 37 °C (Table 2). The synthesis of σ^A in *B. subtilis* DB1005 after a longer exposure to 49 °C was not measured due to the fact that DB1005 was unable to grow at 49 °C and the amount of σ^A in DB1005 decreased within the 120 min interval after the upshift of temperature from 37 to 49 °C in GMM (Chang *et al.*, 1994). All these results indicate that *B. subtilis* DB1005 is unable to sustain the concentration of σ^A at 49 °C. However, it remained unclear whether this was the cause of the temperature sensitivity of *B. subtilis* DB1005.

Fig. 3. Immunoblot analyses of σ^A and GroEL levels in *B. subtilis*. (a) Levels of σ^A and GroEL in *B. subtilis* DB2 and DB1005 before and after temperature elevation. *B. subtilis* strains were grown in 2xSG at 37 °C to an OD₅₅₀ of 0.4 (referred to as time zero) and then shifted to 49 °C. A 1 ml cell sample was harvested at the time points indicated above the panels. After resuspending the cell pellet in 50 μ l 0.5xSET buffer (20% sucrose, 50 mM Tris/HCl pH 7.6, 50 mM EDTA, 2 mM PMSF) and digestion of the cell wall with lysozyme, 50 μ l sample application buffer (Chang & Doi, 1990) was added to rupture the cells. Five microlitres of the cell lysate were then used for immunoblot analysis. (b) Expression of σ^A in *B. subtilis* harbouring plasmids pCX2F or pCX2F5 at 37 °C. *B. subtilis* strains were grown in 2xSG or GMM at 37 °C, harvested at an OD₅₅₀ of 0.4 and treated as in (a). Five microlitres (for 2xSG) or 7 μ l (for GMM) of the cell lysate were used for immunoblot analysis. (c) Levels of σ^A and GroEL in *B. subtilis* harbouring plasmids pCX2F or pCX2F5 before and after temperature elevation. *B. subtilis* strains were grown in GMM at 37 °C to an OD₅₅₀ of 0.4 (referred to as time zero) and then transferred to 49 °C. Cell samples were harvested and treated as in (a). Five microlitres of the cell lysate were used for immunoblot analysis.

Insufficient σ^A concentration is not the cause of the temperature sensitivity of *B. subtilis* DB1005

To verify the relationship between the cellular concentration of σ^A and the temperature sensitivity of *B. subtilis* DB1005, we compared the levels of σ^A in *B. subtilis* DB1005 and DB2 grown in 2xSG medium

Fig. 4. Transcriptional activities of the wild-type and mutant σ^A on the *groE* promoter *in vivo*. (a) Determination of σ^A activity at 37 °C by a *lacZ* transcriptional gene fusion. *B. subtilis* DB2 (●) and DB1005 (▲) cells in which the pCoiZA-derived plasmid (containing the *groE* promoter DNA) has been integrated into the chromosome were grown in 2×SG medium at 37 °C with shaking. Triplicate samples were removed from each culture at the designated OD_{550} and assayed for β -galactosidase activity. Each data point represents the mean value of three parallel samples. (b) Slot blot analysis of σ^A activity at both 37 and 49 °C. Total RNA was extracted from cultures of DB2 and DB1005 grown at 37 °C to an OD_{550} of 0.7 and from the same cultures 10 min after upshifting of the temperature to 49 °C. Ten micrograms of each RNA sample was applied to a Hybond-N membrane with a vacuum blotter and probed with 32 P-labelled *lacZ* DNA prepared by a random priming method. The radioactivity of each sample on the membrane was counted with a radioactivity image reader. The numbers shown in parentheses below the designated temperatures are the ratios of wild-type and mutant σ^A activities obtained by dividing the radioactivity of *lacZ* mRNA in DB2 by that in DB1005.

before and after heat shock. Similar to previous observations (Chang & Doi, 1993b), there was a transient reduction of σ^A in both DB2 and DB1005, but the degree of reduction was quite moderate in DB2. The level of σ^A in *B. subtilis* DB1005 was higher than that in DB2 before the upshift of temperature, which is consistent with the observation that DB1005 has a higher σ^A synthesis rate at 37 °C (Table 2). However, the level of σ^A in DB1005 dropped quite drastically relative to DB2 after a longer

exposure to 49 °C (Fig. 3a). Since DB1005 stopped growing rapidly upon temperature upshift while the levels of σ^A were still high, it seemed that the temperature sensitivity of *B. subtilis* DB1005 was probably not due to insufficient σ^A concentration. To highlight this point, we introduced the pCX2F and pCX2F5 plasmids (see Methods) into *B. subtilis* DB2 and DB1005, respectively, to increase the level of σ^A in the cells. The increase was much more pronounced in 2×SG medium than in GMM for both *B. subtilis* DB2(pCX2F) and DB1005(pCX2F5) at 37 °C (Fig. 3b). However, *B. subtilis* DB1005(pCX2F5) failed to grow in both media after temperature upshift (data not shown), even after the marked increase in the amount of σ^A . In fact, there was an induction of σ^A synthesis in DB1005(pCX2F5) at high temperature and the level of σ^A was much higher in DB1005(pCX2F5) than that in DB2 or in DB2(pCX2F) at parallel time points after temperature elevation (Fig. 3c). The inability of a higher level of σ^A to suppress the Ts phenotype demonstrates that the temperature sensitivity of *B. subtilis* DB1005 is not due to insufficient σ^A concentration.

DB1005 σ^A has a markedly lower activity at 49 °C

We have observed that the accumulation of GroEL in *B. subtilis* DB1005 under heat stress was very slow both in the presence and absence of pCX2F5 (Fig. 3a, c). These results reflected the fact that the mutant σ^A lost a considerable amount of activity after temperature elevation. To examine the relative activity of the mutant σ^A , we transcriptionally fused the *groE* promoter (σ^A -type) (Chang *et al.*, 1994; Li & Wang, 1992; Schmidt *et al.*, 1992) to the *lacZ* gene and integrated this construct into both *B. subtilis* DB2 and DB1005, respectively. Our data showed that the extent of *LacZ* activity was always greater (by about 1.5-fold) in DB2 than in DB1005 during growth at 37 °C (Fig. 4a). Consistent with this finding about 1.77-fold more *lacZ* mRNA was observed in DB2 than in DB1005 at 37 °C at about the same cell density (Fig. 4b). Since β -galactosidase was inactivated at 49 °C, only the amounts of *lacZ* mRNA synthesized in both DB2 and DB1005 at this temperature were compared (Fig. 4b). We found that the *lacZ* mRNA was sharply elevated in DB2 after temperature elevation, whereas it was reduced in DB1005. It was about 5.31-fold higher in DB2 than in DB1005. These results indicate that temperature elevation has a profound adverse effect on the activity of *B. subtilis* DB1005 σ^A .

The effect of temperature elevation on the relative activity of DB1005 σ^A *in vitro* is shown in Fig. 5. No induction of *groE* message was observed for reactions containing the wild-type σ^A above 37 °C. The activities of both wild-type and mutant σ^A factors on the same *groE* promoter peaked at 37 °C and declined as the temperature was elevated (Fig. 5a, c). In addition, there was a slight increase in the ratio of wild-type to mutant σ^A activity (from 3.6 to 4.1) as the reaction temperature was increased from 32 to 49 °C (Fig. 5c). Similarly, the activities of wild-type and mutant σ^A factors on the

Fig. 5. Effect of temperature elevation on transcriptional activity of the wild-type and Ts σ^A *in vitro*. (a, b) Autoradiograms of mRNA transcripts from *in vitro* transcription of (a) *B. subtilis* *groE* promoter and (b) phage $\phi 29$ G3b promoter. The numbers above the panels are the temperatures being tested. The conditions for *in vitro* transcription reactions are given in Methods. (c, d) Relative activities of wild-type (●) and Ts (▲) σ^A factors on (c) *groE* promoter and (d) G3b promoter at various temperatures. The activity of wild-type σ^A at 37 °C was taken as 100%. ✱, Ratio of wild-type to Ts σ^A activity.

strong G3b promoter of *B. subtilis* $\phi 29$ phage peaked at 37 °C. Moreover, the ratio of wild-type to mutant σ^A activity increased from 1.5 to 5.8 as the temperature was raised from 32 to 49 °C (Fig. 5b, d). Therefore, it was clear that the mutant σ^A factor was much less active than the wild-type counterpart at higher temperatures. The correlation between the markedly lower σ^A activity and the temperature sensitivity of *B. subtilis* DB1005 at 49 °C suggests that the level of activity of σ^A is the limiting factor for DB1005 growth at high temperature. Furthermore, the lack of induction of *groE* message *in vitro* in the presence of wild-type σ^A above 37 °C was probably due to the absence of repressors (Yuan & Wong, 1995) in the system, which thus allowed the *groE* promoter to express constitutively.

DISCUSSION

Analyses of the biochemical properties of the mutant σ^A factor in *B. subtilis* DB1005 at both permissive and restrictive temperatures have revealed that it is struc-

turally unstable and easily degraded, even at the permissive temperature. In addition, the transcriptional activity of the mutant σ^A is markedly lower than that of the wild-type counterpart at both temperatures. These results indicate that the two hydrophobic isoleucine residues at positions 198 and 202 are crucial to the structural and functional properties of *B. subtilis* σ^A factor.

Unlike the σ^{70} of *E. coli*, the synthesis of which increases three- to fivefold during heat shock (Taylor *et al.*, 1984), the synthesis of *B. subtilis* σ^A factor was sharply repressed when the cells were subjected to heat shock (Arnosti *et al.*, 1986). Similar to the results reported previously, a transient decrease in σ^A concentration was observed for both *B. subtilis* DB2 and DB1005; however, the process lasted much longer in *B. subtilis* DB1005 than in DB2 (Fig. 3a). The longer duration should not be viewed mainly as the result of rapid degradation of σ^A in DB1005 since the wild-type and mutant σ^A factors have comparable half-lives at high temperature (Table 1).

The most probable cause is the loss of activity of the mutant σ^A whose synthesis is believed to be self-controlled transcriptionally during vegetative growth unless the P1P2 promoter of the *B. subtilis* *sigA* operon is transcriptionally blocked (Hicks & Grossman, 1995). The phenomenon of loss of σ^A activity correlating with transient reduction of protein was also observed for GroEL in DB1005 (Fig. 3a).

σ^A in *B. subtilis* DB1005 was unstable both at permissive and restrictive temperatures (Table 1). However, it was detrimental to the growth of DB1005 only at high temperature. It seems impossible that this phenomenon is due to insufficient σ^A concentration in the cells at 49 °C since overexpression of the mutant σ^A was unable to suppress the Ts phenotype of *B. subtilis* DB1005 (Fig. 3b, c). Other lines of evidence supporting this fact are the presence of a significant amount of σ^A in *B. subtilis* DB1005 before temperature upshift (Fig. 3a), and the failure of a partially improved half-life of the mutant σ^A in the background of major intracellular protease mutation (*isp*) of *B. subtilis* to alleviate the Ts phenotype (Fig. 2, Table 1). Hence, the temperature sensitivity of *B. subtilis* DB1005 must be due to low activity of the mutant σ^A at 49 °C. In other words, the sharp decline in an already reduced activity of DB1005 σ^A might lead to a markedly lower activity of the mutant σ^A at 49 °C (Figs 4, 5), which is probably below a critical level necessary for DB1005 growth. This idea was supported by the restoration of DB1005 growth at high temperature by two different intragenic suppressors of *sigA* through partially enhanced σ^A activity (B.-Y. Chang, unpublished results). Thus, the cessation of DB1005 growth soon after temperature upshift (37 to 49 °C) might be the symptom of the adverse effect of high temperature on the activity of DB1005 σ^A , while the resumption of growth after temperature downshift (49 to 37 °C) might indicate the elimination of this adverse effect. Consequently, we conclude that the temperature sensitivity of *B. subtilis* DB1005 is mainly due to low activity rather than insufficient concentration of σ^A in DB1005 at high temperature. Therefore, the nature of the Ts mutation in *B. subtilis* DB1005 is evidently different from that of the *E. coli* *rpoD* 800 Ts mutant. The Ts phenotype of this *E. coli* strain can be ascribed to insufficient σ^{70} concentration rather than altered function of the σ^{70} protein, since it can be suppressed by mutations in the *lon* protease gene (Grossman *et al.*, 1983; Liebke *et al.*, 1980).

Other important observations in our studies are the compensation of σ^A in *B. subtilis* DB1005 at 37 °C (Table 2) and the induction of σ^A synthesis in *B. subtilis* DB1005(pCX2F5) within 5 min after temperature upshift (Fig. 3a). The increased level of σ^A at 37 °C was also observed in two other *sigA* mutants in which proline or glycine residues had been introduced into the promoter -10 binding region of σ^A (B.-Y. Chang, unpublished results). It seems that there is an unknown autogenous control of the level of σ^A in *B. subtilis*. One possibility is that this control is carried out by transcription from promoters other than P1P2 on the *sigA* operon, as

reported for the *csb203::Tn917lac* mutant of *B. subtilis* in which the minor σ factor σ^H becomes essential for the expression of σ^A and for vegetative growth (Hicks & Grossman, 1995). The induction of σ^A synthesis in *B. subtilis* DB1005(pCX2F5) at 49 °C was unexpected since the mutant σ^A lost most of its activity at the elevated temperature (Figs 4, 5) and no such induction was observable in *B. subtilis* DB1005 under heat stress (Fig. 3a). Most likely, the induction is closely related to the expression of plasmid-borne 'mutant σ^A ' in *B. subtilis* DB1005(pCX2F5), otherwise similar induction should also be observed in DB2(pCX2F) under the same conditions (Fig. 3c). The mechanism of σ^A induction in DB1005(pCX2F5) at the elevated temperature and its relation, if any, to the regulation of the *sigA* gene in *B. subtilis* DB1005 remain unclear. Further experiments are needed to answer these questions.

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