Regulatory role of RsgI in *sigI* expression in *Bacillus subtilis*

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The sigma gene, *sigI*, of *Bacillus subtilis* belongs to the group IV heat-shock response genes and has many orthologues in the bacterial phylum Firmicutes. The *B. subtilis sigI* gene is considered to constitute an operon with *rsgI* (regulation of *sigI*, formerly *ykrl*). As little is known about either the structure and function of the *sigI-rsgI* operon or the SigI regulons, the role of RsgI in heat-inducible transcription of the *sigI-rsgI* operon was investigated, using Northern analysis and a heat-stable β -galactosidase reporter assay. Heat-inducible, SigI-dependent transcription of the *sigI-rsgI* operon was stimulated greatly by disrupting *rsgI*. Yeast two-hybrid analysis showed direct interaction between the N-terminal portion of the presumed RsgI protein and SigI. Without RsgI function, induction of transcription of the *sigI-rsgI* operon upon transient heat stress depended on *dnaK* activity. However, transcription of the operon was induced during growth at prolonged higher temperature even without DnaK function. Without RsgI function, *sigI-rsgI* operon transcription was induced after the end of growth independent of any temperature shift in a sporulation medium and toward the end of growth in a rich complex medium. Furthermore, glucose addition resulted in a strong suppression of *sigI-rsgI* transcription. Therefore it is hypothesized that transcription of the *sigI-rsgI* operon of *B. subtilis* is negatively regulated by the putative transmembrane protein RsgI, which moderates SigI's sensitivity to heat shock or nutritional stress.

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INTRODUCTION

Bacterial response to transient stresses is mediated largely through transcription of relevant genes directed by sigma factors (Missiakas & Raina, 1998; Wösten, 1998; Raivio & Silhavy, 2001; Helmann, 2002; Mittenhuber, 2002). These include the heat-shock factor RpoH and stationary-phase protein RpoS of *Escherichia coli* (Morita *et al.*, 1999; Weber *et al.*, 2005), the general stress-response protein SigB of *Bacillus subtilis* (Peterson *et al.*, 2001; Price *et al.*, 2001; Price, 2002) and ECF (extracytoplasmic function) sigmas such as SigE of *Streptomyces coelicolor*, RpoE of *E. coli* and SigW of *B. subtilis* (Lonetto *et al.*, 1994; Raina *et al.*, 1995; Rouviere *et al.*, 1995; De La Penas *et al.*, 1997; Wiegert *et al.*, 2001).

These sigma factors usually exist at low concentrations or in an inactive form. Upon stress, in most cases, activation of these sigma factors and subsequent autoregulatory induction of transcription of their own genes leads to sufficient concentrations of sigma factor to transcribe their regulons. A translational activation process is involved in the case of RpoH of *E. coli* (Morita *et al.*, 1999).

Some of the stress-responding sigma genes are associated with anti-sigma factor genes, whose products inhibit the activity of

sigma factors through direct binding, which is disrupted upon stress through phosphorylation by anti-anti-sigma factors or digestion by endogenous proteases (Hughes & Mathee, 1998).

The *sigI* (*ykoZ*) operon of *B. subtilis* is thought to consist of *sigI* and *rsgI* (regulation of *sigI*, formerly *ykrl*) (Kunst *et al.*, 1997), which encodes a σ^{70} -type sigma factor, SigI, and a putative membrane protein, RsgI. Transcription of the *sigI* gene is induced by heat shock in a SigI-dependent manner, and the wild-type *sigI* gene is required for the growth at higher temperatures (Zuber *et al.*, 2001). However, the role of *rsgI* is not known and little is known about the SigI regulons. As there is not a group I specific CIRCE sequence, a group II specific SigB-dependent promoter, or a group III specific CtsR-binding site within the upstream region of *sigI*, the *sigI* gene is proposed to be a member of group IV of the *B. subtilis* heat-shock genes (Zuber *et al.*, 2001).

Orthologues (see <http://bacillus.genome.jp/>) of SigI and RsgI of *B. subtilis* are found in many species of the phylum Firmicutes. Their function in these organisms remains to be elucidated.

In this work, we studied the role of RsgI in the response to heat stress and in the expression of the *sigI* operon. To do

this we introduced a heat-stable reporter gene into *B. subtilis* by constructing a strain carrying at the *amyE* locus the *bgaB* gene from *Bacillus stearothermophilus* encoding a heat-stable β -galactosidase fused to the promoter of the *sigI* operon (Hirata *et al.*, 1986; Yuan & Wong, 1995). Expression of *bgaB* and amounts of transcript of the *sigI* operon were examined in mutant strains with disrupted *sigI* or *rsgI* genes.

METHODS

Strains, plasmids, DNA manipulation and culture media. All strains and plasmids used in this study are described in Table 1. Cells of *B. subtilis* or *E. coli* were grown in Luria–Bertani broth or in a complex sporulation medium (Sambrook *et al.*, 1989; Schaeffer *et al.*, 1965). DNA manipulation, PCR, reagents and enzymes were as described elsewhere (Miwa *et al.*, 2000; Ohshima *et al.*, 2002).

Construction of the *B. subtilis* strain carrying at the *amyE* locus the thermostable *bgaB* gene fused to the promoter of the *sigI* operon. A portion of the promoter region of the *sigI* gene was synthesized by PCR using template DNA from a wild-type *B. subtilis* strain and the primer pair 5'-GAAGAATTCTGGGGTGTCTTAGCAG-3'/5'-GGAGGATCCACTGGTTTCACCTCAGTTTC-3'. DNA was digested with *EcoRI* and *BamHI*, and cloned into plasmid pDLd, which carries the heat-stable *bgaB* gene of *B. stearothermophilus* integrated into the *amyE* locus on the plasmid (Hirata *et al.*, 1986; Yuan & Wong, 1995). The sample was used to transform wild-type strain 168 to chloramphenicol resistance, to obtain a strain carrying at the *amyE* locus the *bgaB* gene with the promoter of the *sigI* operon in the plasmid pDLd backbone. The resulting gene fusion was designated *amyE::P_{sigI}-bgaB*.

BgaB activity. Heat stable β -galactosidase activity of the *bgaB* gene from *B. stearothermophilus* was measured at 62 °C as described elsewhere (Yuan & Wong, 1995; Platt *et al.*, 1972; Sadaie, 1998).

Table 1. Bacterial strains and plasmids

Strain or plasmid	Genotype and/or relevant phenotype	Reference and/or source
<i>B. subtilis</i>		
168	<i>trpC2</i>	Laboratory stock
BSU11	<i>trpC2 sigI::pMutinT3 Em^r</i>	This work
BSU12	<i>trpC2 rsgI::pMutinT3 Em^r</i>	This work
BSU13	<i>trpC2 rsgI::em Em^r</i>	This work
BSU14	<i>trpC2 dnaK::sp Sp^r</i>	H. Yoshikawa*
BSU15	<i>trpC2 amyE::P_{sigI}-bgaB Cm^r</i>	This work
BSU16	<i>trpC2 amyE::P_{sigI}-bgaB rsgI::pMutinT3 Cm^r Em^r</i>	This work
BSU17	<i>trpC2 amyE::P_{sigI}-bgaB rsgI::em Cm^r Em^r</i>	This work
BSU18	<i>trpC2 amyE::P_{sigI}-bgaB sigI::pMutinT3 Cm^r Em^r</i>	This work
BSU19	<i>trpC2 amyE::P_{sigI}-bgaB sigI::pMutinT3 rsgI::pMutinT3::sp Cm^r Em^r Sp^r</i>	This work
BSU20	<i>trpC2 amyE::P_{sigI}-bgaB dnaK::sp Cm^r Sp^r</i>	This work
BSU21	<i>trpC2 amyE::P_{sigI}-bgaB rsgI::pMutinT3 dnaK::sp Cm^r Em^r Sp^r</i>	This work
BSU22	<i>trpC2 amyE::P_{sigI}-bgaB rsgI::em dnaK::sp Cm^r Em^r Sp^r</i>	This work
BSU23	<i>trpC2 amyE::P(-35)_{sigI}-bgaB Cm^r</i>	This work
BSU24	<i>trpC2 amyE::P(-10)_{sigI}-bgaB Cm^r</i>	This work
BSU25	<i>trpC2 amyE::P(+1)_{sigI}-bgaB Cm^r</i>	This work
BSU26	<i>trpC2 sigI::em</i>	This work
BSU27	<i>trpC2 sigI-rsgI::em</i>	This work
BSU28	<i>amyE::P_{sigI}-bgaB rsgI::em spo0AΔHB</i>	This work
<i>E. coli</i>		
C600	<i>thi-1 thr-1 leuB6 lacY1 tonA21 supE44</i>	Laboratory stock
JM109	<i>recA1 edbA1 gyrA96 thi-1 hsdR17 supE44 Δ(lac-proAB)F'(traD36 proAB⁺ lacI^f)</i>	Laboratory stock
<i>S. cerevisiae</i>		
PJ69-4A	<i>MATa, trp1-901, leu2-3, 112 ura3-52, his-200, gal4Δ gal80Δ, LYS2::GAL1-HIS3, GAL2-ADE2, met2::GAL7-lacZ</i>	Yoshimura <i>et al.</i> (2004)
PJ69-4α	<i>MATα, trp1-901, leu2-3, 112 ura3-52, his-200, gal4Δ, gal80Δ, LYS2::GAL1-HIS3, GAL2-ADE2, met2::GAL7-lacZ</i>	Yoshimura <i>et al.</i> (2004)
Plasmids		
pDLd	<i>amyE::bgaB Ap^r Cm^r</i>	Laboratory stock
pADT7	<i>GAL4AD LEU2 Ap^r</i>	Clontech
pBTK	<i>GAL4DNA-BD TRP1 Km^r</i>	Clontech
pEr::Sp	<i>Sp^r</i>	Lindow <i>et al.</i> (2002)

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Samples were heated at 68 °C for 5 min before measuring BgaB activity, to inactivate the endogenous β -galactosidase of the *lacZ* gene of the *E. coli* in the integrated pMutin plasmid.

Gene disruption. Disruption of *sigI* and *rsgI* was performed as described elsewhere, with pMutin3 carrying DNA corresponding to a portion of each gene (Vagner *et al.*, 1998). The DNA fragment cloned was obtained by PCR synthesis with a template DNA from a wild-type strain and primer pairs 5'-AAGAAGCTTCCAGTGCTT-AGCCTTTTG-3'/5'-GGAGGATCCATTTTGTGCGCTTCTGGC-3' for *sigI*, and 5'-AAGAAGCTTCGTACAGTTGCTGACCC-3'/5'-GGAGGATCCATTTTCGACGCTTGGATTG-3' for *rsgI*. Gene disruption with the Em cassette was performed as follows. Both side regions of the target gene were PCR amplified with a template DNA from a wild-type strain and primer pairs described below. The resulting DNA fragments were subjected to PCR amplification with a DNA carrying the *em* gene of pMutin to obtain a DNA fragment that contained *em* gene instead of the target gene. The resulting DNA was used to transform wild-type *B. subtilis* to erythromycin resistance to obtain an *em*-substituted strain. For *sigI* disruption, we used two primer pairs, 5'-TTGCGGCTTCCATGTTTGT-3'/5'-GCACTATCAACACACTCTTAAGTTATGCAGATCTTTATTGCCTTTTG-3' and 5'-GGAGCTAAAGAGGTCCCTAGGTGTATTATCATTACGGGTG-3'/5'-CTCAGAGATTCCATGCGG-3'. For *rsgI* disruption, the primer pairs used were 5'-GACGGTTTCATCGTTTG-3'/5'-GCACTATCAACACACTCTTAAGTTATCTTCTCA-TGAGTGCAGC-3' and 5'-GGAGCTAAAGAGGTCCCTAGCCG-GCGAATAAAGACCTG-3'/5'-CTCAGACAGATCTGATGA-3'. For *sigI-rsgI* disruption, the primer pairs 5'-TTGCGGCTTCCA-TGTTTGT-3'/5'-GCACTATCAACACACTCTTAAGTTATGCAGATCTTTATTGCCTTTTG-3' and 5'-GGAGCTAAAGAGGTCCCTAG-CCGGCGAATAAAGACCTG-3'/5'-CTCAGACAGATCTGATGA-3' were used. Underlined portions of the primers possess the same sequences as the 5' or 3' portion of the *em* gene of pMutin, which was amplified using pMutin DNA and the primer pair 5'-CTTAAGAGTGTGTTGATAGTGC-3'/5'-CTAGGGACCTCTTAG-CTCC-3'. The amplified 948 bp *em* gene contained the promoter sequence (−35, −10), SD sequence, and the *em* structural gene (encoding a 245 aa protein).

Construction of pMutinT3::sp insertional disruptant. The *em* gene of pMutin was substituted with the *sp* gene of plasmid pEr::sp (Lindow *et al.*, 2002) by transformation of pMutin insertional disruptant BSU19 to Sp^r with linearized pEr::sp DNA.

Construction of BSU28 (*rsgI::em spo0AΔHB*). BSU17 was transformed to Trp⁺ Spo0A[−] with DNA from a strain that is a His⁺ Spo0A[−] transformant of NIG1121 (*met his*) (Sadaie, 1998) with DNA from UOT1611 (*trpC2 lysI aprEΔ3 nprE18 nprR2 spo0AΔHB*) (Asai *et al.*, 1995).

Deletion of the promoter region of the *sigI-rsgI* operon. The *sigI* promoters with deletions were made by fusing to the *bgaB* gene, in the *amyE* locus on the pDLd plasmid, DNA fragments PCR-synthesized using template DNAs from a wild-type strain and the primer pairs 5'-GAAGAATTCAAAAACACGCATAAAACCCCC-3'/5'-GGAGGATCCACTGGTTTACCTCAGTTC-3' for the −35 deletion, 5'-GAAGAATTCTAGAAAGGCAGAAATCATGT-3'/5'-GGA-GGATCCACTGGTTTACCTCAGTTC-3' for the −10 deletion, and 5'-GAAGAATTCAGAACGTCAGAAATGGTTTGTG-3'/5'-GGAGGA-TCCCTGGTTTACCTCAGTTC-3' for the +1 deletion.

Conditions for primer extension and sequencing reactions. RNA was prepared from cells of strains BSU15 (*P_{sigI}-bgaB*), BSU14 (*P_{sigI}-bgaB rsgI*), BSU17 (*P_{sigI}-bgaB sigI*) or BSU19 (*P_{sigI}-bgaB sigI rsgI*) grown at 37 °C or 50 °C. The primer extension reaction was carried out as described elsewhere with digoxigenin-labelled primer DNA (10 pmol, 5'-CAAATTGAGGATAACACATTC-3', a sequence

complementary to the region underlined with an arrow in Fig. 4) and reverse transcriptase (Invitrogen SuperScript II RNase H[−] Reverse Transcriptase) (Takamatsu *et al.*, 2000). Sequencing of the *sigI* promoter region was performed with the DIG-labelled primer DNA described above and PCR-synthesized template DNA containing the promoter region of the *sigI-bgaB* operon. The latter DNA was PCR-synthesized with a template DNA from BSU15 (*P_{sigI}-bgaB*) and the primer pair 5'-GAAGAATTCTGGGGTGTCTTAGCAG-3'/5'-CCTTGTCTAGCCATTCAAAG-3' (shown as dotted arrows in Fig. 4). The PCR product was purified by electrophoresis.

RNA isolation. Total RNA was extracted from cells resuspended in a buffer containing phenol and glass beads by procedures essentially similar to those described elsewhere (Asai *et al.*, 2003).

Northern analysis. This was performed as described elsewhere (Ohshima *et al.*, 2002).

Cloning of *sigI*, *rsgI* and *sigA* into pGADT7 and pGBTK for yeast two-hybrid analysis. Yeast two-hybrid analysis was performed as described elsewhere (Yoshimura *et al.*, 2004). Plasmid vectors pGBTK and pGADT7 carrying cloned *B. subtilis* genes were used to transform mating-type yeast strains PJ69-4A and PJ69-4 α , respectively. The *sigI*, *sigA* and *rsgI* genes, the 5'-terminal portion of *rsgI* and the 3'-terminal portion of *rsgI* were cloned into pGADT7 or pGBTK by inserting PCR-synthesized DNA fragments. PCR was performed with template DNA from a wild-type strain and the following primer pairs: 5'-GAAGAATTCATGAAACCAGTGCTTAGC-C-3'/5'-GGAGGATCCTCATGAGTGCAGCACCCC-3' for *sigI*, 5'-GGAGGATTCATGAGAAGAGGGATTATAG-3'/5'-GGAGGATCCT-TATTCGCCGGGGGCACTC-3' for *rsgI*, 5'-GGAGGATTCATGA-GAAGAGGGATTATAG-3'/5'-GGAGGATCCTTAGCGCAGTTTAA-AAAAATCAAAAAAG-3' for the *rsgI* N-terminal portion, 5'-GGAGGATTCATGCGTATATGACAATCG-3'/5'-GGAGGATCCT-TATTCGCCGGGGGCACTC-3' for the *rsgI* C-terminal portion, and 5'-GAAGAATTCATGGCTGATAAACAAACCC-3'/5'-GGAGGATC-CTTATTCAAGGAAATCTTTCAAACG-3' for *sigA*.

RESULTS

Heat induction of transcription of the *sigI* gene of *B. subtilis* is elevated greatly by disruption of the downstream *rsgI* gene

Northern blot analysis of the *sigI* gene of *B. subtilis* revealed that its transcription is inducible upon heat shock (Zuber *et al.*, 2001). We confirmed the heat-inducibility of transcription of *sigI* by observing expression of the heat-stable BgaB activity of the *bgaB* gene of *B. stearothermophilus* fused to the *sigI* promoter (*P_{sigI}-bgaB*) at the *amyE* locus (see Methods). Cells of *B. subtilis* strains carrying the *P_{sigI}-bgaB* fusion were grown in LB medium at 37 °C and transferred to 50 °C. Induction of BgaB activity in the wild-type strain BSU15 was not observed following transient heat shock for 15 min at 50 °C (Fig. 1a), but was observed during sustained growth at 50 °C (Fig. 1b). Disruption of the *rsgI* gene by *em* displacement (*rsgI::em*) in strain BSU17, on the other hand, stimulated greatly the expression of BgaB activity by transient heat shock (Fig. 1a) as well as in sustained growth at the higher temperature (Fig. 1b).

Transcription of the *sigI* gene depends on SigI

The induction of BgaB activity by transient heat shock was confirmed in the *rsgI*-insertionally disrupted strain BSU16

Fig. 1. Heat induction of *sigI-rsgI* operon transcription is stimulated by disruption of the *rsgI* gene and dependent on SigI. The upper panels show the growth curve of each strain and the lower panels show the BgaB activity. (a) Cells were grown at 37 °C, transferred to 50 °C at time 0, and returned to 37 °C after 15 min. BgaB activity from P_{sigI} -*bgaB* was measured in the wild-type strain BSU15 (●, ○) and *rsgI*::*em*-disrupted strain BSU17 (▼, ▽). Filled symbols, heat-shocked cells; open symbols, cells incubated at 37 °C without heat shock. (b) Cells were grown at 37 °C, then transferred to 50 °C at time 0. BgaB activity from P_{sigI} -*bgaB* was measured in the wild-type strain BSU15 (●, ○) and *rsgI*::*em*-disrupted strain BSU17 (▼, ▽). Filled symbols, temperature-shifted cells; open symbols, cells incubated at 37 °C without temperature shift. (c) Cells were grown at 37 °C and transferred to 50 °C for 15 min as described in (a). Activity of BgaB from P_{sigI} -*bgaB* was measured in the *rsgI*::pMutinT3-disrupted strain BSU16 (▼, ▽) and the doubly disrupted *rsgI*::pMutinT3::sp *sigI*::pMutinT3 strain BSU19 (■, □). Filled symbols, heat-shocked cells; open symbols, cells incubated at 37 °C without heat shock.

(*rsgI*::pMutinT3) (Fig. 1c), but was not observed when both *sigI* and *rsgI* were disrupted in strain BSU19 (*rsgI*::pMutinT3::sp *sigI*::pMutinT3) (Fig. 1c). Furthermore, an increase in BgaB activity during sustained growth at higher temperature (50 °C) was not observed in the *sigI*-disrupted strain (data not shown in Fig. 1b). An independent experiment showed β -galactosidase activity of ~4.6 Miller units in the *sigI*-disrupted strain. This was also confirmed in the primer extension experiment described in a later section. Therefore transcription of the *sigI* gene is SigI-dependent, i.e. auto-regulated.

Detection of the mRNA of the complete *sigI-rsgI* operon

Heat-shock induction of *sigI* was confirmed by Northern blot analysis of RNA extracted from cells treated with heat. The expected transcript of more than 1896 nt from the predicted *sig-rsgI* operon was not detected upon transient heat shock (50 °C for 15 min) in the wild-type strain 168 (Fig. 2a). However, heat shock induced greatly a transcript hybridizing to the *sigI* probe in the *rsgI*-disrupted (*rsgI*::*em*) strain BSU13 (Fig. 2b). The sizes of the wild-type *sigI* and *rsgI* genes are 752 nt and 1142 nt respectively, and the *em*-substituted disrupted *rsgI* gene is 967 nt long. The expected size of the transcript from the *sigI-rsgI*::*em* gene is slightly longer than 1719 nt. The heat-induced signal detected

showed a transcript of ~1500 nt, which is longer than the 752 nt transcript expected from *sigI* or the 967 nt transcript expected from *rsgI*::*em*. Thus, the signal detected must be the transcript from the entire *sigI-rsgI* operon, possibly quickly processed to give a shorter mRNA.

The start site of transcription of the *sigI-rsgI* operon

The transcription start site was determined by reverse transcription of the mRNA derived from P_{sigI} -*bgaB* at the *amyE* locus. An oligonucleotide complementary to the *bgaB* region was used for primer extension. Experiments using RNA derived from cells of the wild-type strain BSU15 or the *rsgI*-disrupted (*rsgI*::*em*) strain BSU17 grown in LB medium at 37 °C or 50 °C revealed a unique transcription initiation site at a G residue located 116 bases upstream of the presumed initiation codon (GTG) of the *sigI* gene (Fig. 3, lanes 1–4, and Fig. 4). Experiments using cells of both strains grown at the higher temperature gave stronger signals (Fig. 3, lanes 2 and 4) than those from cells grown at the lower temperature (Fig. 3, lanes 1 and 3). The *rsgI*-disrupted strain showed a slightly stronger signal (Fig. 3, lanes 1 and 2) than the wild-type strain (Fig. 3, lanes 3 and 4). This transcription was SigI-dependent, as the *sigI* disruptant BSU18 (*sigI*::pMutinT3) and the *sigI-rsgI* double disruptant strain BSU19 (*sigI*::pMutinT3

Fig. 2. Northern analysis of the transcript of the *sigI-rsgI* operon of the wild-type strain 168 (a) and the *rsgI::em*-disrupted strain BSU13 (b). RNA was extracted from the cells after heat treatment at 50 °C for 15 min. An RNA probe hybridizable to *sigI* was used. Numbers indicate minutes after heat shock. Arrows on the right indicate the positions of size markers: 3, 2.5, 2, 1.5 and 1 kb.

rsgI::pMutinT3::sp) gave no signals at either temperature (Fig. 3, lanes 5, 6, 9 and 10).

The promoter sequences of the *sigI-rsgI* operon were assumed to be ACCCCC for the -35 region and AAATC for the -10 region (Fig. 4).

Deletion analysis of the promoter region was performed to confirm identification of the above transcription initiation site, because a paper reported that transcription of the *sigI* operon starts from two different sites, 18 and 23 bases downstream of the initiation site described above (Zuber *et al.*, 2001). Deletion of the upstream region of the promoter (BSU23) did not strongly reduce *sigI* operon

Fig. 3. Primer extension analysis to determine the transcription initiation site of the *sigI-rsgI* operon. mRNAs were isolated from cells of P_{sigI} -*bgaB* *rsgI::em* strain BSU17 (lanes 1 and 2), P_{sigI} -*bgaB* strain BSU15 (lanes 3 and 4), P_{sigI} -*bgaB* *sigI::pMutinT3* strain BSU18 (lanes 5 and 6), and P_{sigI} -*bgaB* *sigI::pMutinT3* *rsgI::pMutinT3::sp* doubly disrupted strain BSU19 (lanes 9 and 10) grown at 50 °C (lanes 2, 4, 6 and 10) or at 37 °C (1, 3, 5 and 9). No sample was present in lanes 7 and 8. DIG-labelled primer hybridizable to *bgaB* was used.

transcription (Figs 4 and 5), while deletion of the -35 region (BSU24) resulted in a background level of transcription, i.e. similar to transcription in the *sigI*-disrupted strain. Deletion of the -35 region should not result in reduced transcription if transcription starts 18 or 23 bases downstream of the predicted start site. Therefore our deletion analysis seemed to support the transcription start site

Fig. 4. DNA sequence of the *sigI* promoter region fused with the *bgaB* gene and transcription initiation site. The G marked with * indicates the transcription initiation site determined in this study. The -35 and -10 sequences are underlined; the long solid arrow indicates the sequence complementary to the primer used in determination of the transcription initiation site. Slashes with #1, #2, and #3 indicate the boundary of downstream regions that were cloned to produce the $P(-35-10)_{sigI}$ -*bgaB*, $P(-10)_{sigI}$ -*bgaB* and promoterless *bgaB* fusions. Initiation codons for *sigI* (GTG) and *bgaB* (ATG) are indicated. Dotted underlining indicates the *cre*-like sequence. Converging arrows indicate the transcription termination signal sequence of *ykoY*. Dotted vertical lines indicate the promoter region cloned and fused to the *bgaB* gene. Dotted arrows indicate a primer pair (see text).

Fig. 5. Effects on promoter activity of deletions in the promoter region. The upper panel shows the growth curve of each strain and the lower panel shows the BgaB activity. Rapidly growing cells at 37 °C were transferred to 50 °C at time 0. BgaB activity was measured for strains carrying wild-type P_{sigI} - $bgaB$ (BSU15, black circles), $P(-35-10)_{sigI}$ - $bgaB$ (BSU23, dark grey circles, #1), $P(-10)_{sigI}$ - $bgaB$ (BSU24, light grey circles, #2) or promoterless fusion (BSU25 open circles, #3). #1, #2 and #3 are described in the legend for Fig. 4. Open triangles indicate BgaB activity from wild-type promoter fusion P_{sigI} - $bgaB$ in the $sigI::pMutinT3$ -disrupted strain BSU16.

determined in this study. Deletion of the -35 and -10 sequences completely eliminated transcription from the *sigI* promoter.

Yeast two-hybrid analysis of SigI and RsgI

To test whether or not RsgI directly interacts with SigI, we carried out a yeast two-hybrid analysis. As shown in Fig. 6, the full-length SigI and RsgI proteins interacted when SigI protein was fused to the DNA-binding domain and RsgI protein was fused to the activation domain. Furthermore, the full-length SigI protein and N-terminal portion (1st to 58th aa) of the RsgI protein interacted directly, as fusions either to the binding domain or to the activation domain. On the other hand, SigI did not show interaction with the C-terminal domain (90th to the final 381st aa) of RsgI. As RsgI is assumed to possess one transmembrane domain (67th to 89th aa predicted by SOSUI: <http://bp.nuap.nagoya-u.ac.jp/sosui/>), our results suggest that the cytoplasmic N-terminal portion (1st to 58th aa) of the RsgI protein must interact directly with the SigI protein. We also found that SigA did not interact directly with RsgI.

Fig. 6. Yeast two-hybrid analysis of direct interactions between SigI and the N-terminal portion of RsgI. Bait strain PJ69-4A carried pGBTK or its derivative plasmid, and prey strain PJ69-4A carried pGADT7 or its derivative plasmid. Diploid strains were constructed by mating these two strains. AD, activation domain; BD, DNA-binding domain.

DnaK is required for the SigI response to transient heat stress

The above results suggested that RsgI negatively regulated SigI activity by direct interaction. However, heat shock was required to activate SigI, even in the absence of RsgI. Heat activation may involve a chaperone. Disruption of both *dnaK*, a member of the chaperone gene family (Homuth *et al.*, 1999), and *rsgI* in strain BSU22 (*dnaK::sp rsgI::em*) resulted in no stimulation (for 30 min) of the transient heat response of the *sigI*-*rsgI* operon which was observed in the *rsgI*-disrupted strain BSU17 (*rsgI::em*) (Fig. 7a). However, prolonged heat induced *sigI*-*rsgI* operon transcription even in the *dnaK rsgI* double disruptant BSU22 (Fig. 7b). Disruption of *rsgI* by the insertional plasmid pMutinT3 gave the same result (data not shown). These results suggest that transient heat activation of SigI depends upon DnaK.

Induction of *sigI*-*rsgI* operon transcription in the *rsgI*-disrupted strain during growth in a complex sporulation medium, and near the end of growth in LB broth

To elucidate what signals other than heat induce transcription of the *sigI*-*rsgI* operon, we examined *sigI* operon transcription during sporulation. As shown in Fig. 8(a), BgaB activity resulting from P_{sigI} - $bgaB$ at the *amyE* locus increased 1 h after the onset of sporulation in the wild-type strain BSU15 or the *rsgI* disruptant BSU17 (*rsgI::em*) grown at 37 °C in a complex sporulation medium. This activity was

Fig. 7. DnaK-dependent activation of heat-shock-induced *sigI-rsgI* operon transcription. The upper panels show the growth curve of each strain and the lower panels show the BgaB activity. (a) Rapidly growing cells at 37 °C were transferred at time 0 to 50 °C for 15 min, and returned to 37 °C as described in the legend for Fig. 1(a). BgaB activity was measured for the *bgaB* gene fused to the *sigI* promoter in BSU17 (*rsgI::em*, ▼, ▽) and BSU22 (*rsgI::em dnaK::sp*, ◆, ◇). Filled symbols, heat-shocked cells; open symbols, cells incubated at 37 °C without heat shock. (b) Rapidly growing cells were transferred from 37 °C to 50 °C at time 0. BgaB activity was measured for the *bgaB* gene fused to the *sigI* promoter in BSU16 (*rsgI::pMutinT3*, ▼, ▽) and BSU21 (*rsgI::pMutinT3 dnaK::sp*, ◆, ◇). Filled symbols, temperature-shifted cells; open symbols, cells incubated at 37 °C without temperature shift.

dependent on SigI, as the *sigI-rsgI* double disruptant strain BSU19 (*sigI::pMutinT3 rsgI::pMutinT3::sp*) did not show such an increase (data not shown). Disruption of the *dnaK* gene in addition to *rsgI* disruption in BSU21 (*rsgI::pMutin dnaK::sp*) delayed the start of induction of BgaB activity by about 60 min (data not shown). DnaK must be required toward the end of growth as well as for the response to heat stress, to activate free SigI protein in the *rsgI* disruptant strain.

Sporulation is induced by phosphorylation of Spo0A protein, followed by derepression of *sigH* through repression by Spo0A-P of AbrB expression (AbrB is the repressor of the *sigH* gene, which encodes the first sigma factor during sporulation: Stragier & Losick, 1996). SigI expression may be induced by direct activation of free SigI protein induced by phosphorylation of Spo0A protein, or by derepression or activation of the *sigI* promoter by spo0A-P, AbrB or SigH. However, in the *spo0A* mutant strain BSU28 (*rsgI::em spo0A*), we did not observe any reduction of the above increase of BgaB expression from P_{*sigI*}-*bgaB* upon sporulation (Fig. 8a). Therefore it was considered that *sigI* induction is not related to sporulation.

Activation of *sigI-rsgI* operon transcription in strain BSU15 or BSU17 (*rsgI::em*) was also induced toward the end of growth even at 37 °C in LB broth (Fig. 8b), which does not support efficient sporulation. Induction was transient and showed a maximal value near the end of growth. This

induction depended on SigI, because the *rsgI* disruptant (BSU17) showed strong induction but the *rsgI sigI* double disruptant BSU19 (*sigI::pMutinT3 rsgI::pMutinT3::sp*) showed reduced induction (data not shown). Furthermore, this induction was not dependent on DnaK, as the *rsgI* and *dnaK* double disruptant BSU21 (*rsgI::em dnaK::sp*) showed the same induction as BSU17.

Glucose repression of *sigI-rsgI* operon expression

Addition of glucose strongly suppressed activation of *sigI-rsgI* operon transcription in strain BSU17 (*rsgI::em*) grown at 37 °C in a complex sporulation medium (Fig. 8a) or in strain BSU15 grown at 50 °C in LB broth as described below. Glucose causes repression of gene expression by CcpA through the catabolite fructose biphosphate (Deutscher *et al.*, 2002). Although the CcpA target-like sequence (complementary *cre*-like sequence of ATGAAAACGCTTCAA) was found upstream of the -35 region (Fig. 4) (Miwa *et al.*, 2000), deletion of this target sequence resulted in glucose-sensitive *sigI-rsgI* expression as shown by strain BSU23. Rapidly growing cells of BSU15(P_{*sigI*}-*bgaB*) or BSU23(P(-35)_{*sigI*}-*bgaB*) in LB broth at 37 °C were transferred to 50 °C for 60 min with or without glucose (0.5 %) addition. BSU15 showed BgaB activity of 16.5 Miller units (MU) without glucose and 3.5 MU with glucose, while BSU23 showed BgaB activity of 6.2 MU without glucose and 1.7 MU with glucose.

Fig. 8. Induction of the SigI-dependent transcription of the *sigI-rsgI* operon during sporulation or at the end of the exponential phase of growth in LB medium. The upper panels show the growth curve of each strain and the lower panels shows the BgaB activity. (a) Cells were grown in Difco sporulation broth at 37 °C. (b) Cells were grown in LB medium at 37 °C. BgaB activity was measured for the *bgaB* gene fused to the *sigI* promoter in strains BSU15 (P_{sigI} -*bgaB*, ○), BSU17 (P_{sigI} -*bgaB* *rsgI*::*em*, ▽), BSU22 (P_{sigI} -*bgaB* *rsgI*::*em dnaK*::*sp*, ◇) or BSU28 (P_{sigI} -*bgaB* *rsgI*::*em spo0A*Δ*HB*, □). ▼, Growth and BgaB activity of BSU17, cells of which were grown at 37 °C in Difco sporulation medium containing 0.5 % glucose.

DISCUSSION

Our results both confirmed some of the previous findings and revealed some new features of the structure and function of the *sigI-rsgI* operon of *B. subtilis*. Since transcription of the *sigI* gene is inducible by heat, construction of a heat-stable β -galactosidase gene (*bgaB*) fused to the *sigI* promoter enabled measurement of transcription of the *sigI-rsgI* operon. Furthermore, reverse transcription of transcripts derived from P_{sigI} -*bgaB*, using a primer complementary to *bgaB*, allowed determination of a unique, heat-inducible and SigI-dependent transcription initiation site.

The predicted RsgI protein has a transmembrane sequence, and a long C-terminal sequence containing an aspartic-acid-rich region (BSORF: <http://bacillus.genome.jp/>). The N-terminal portion (first 58 aa excluding transmembrane domain) of RsgI interacted with SigI. Deletion or pMutin-insertional inactivation of the *rsgI* gene resulted in effects on heat-inducible SigI activation. The latter insertion left the N-terminal sequence including the transmembrane sequence intact. Therefore, even if SigI-interacting sequences and the transmembrane sequence of RsgI were retained *in vivo*, the SigI-interacting sequence did not function. This suggests

that RsgI requires the long C-terminal domain to negatively regulate SigI factor, or to be properly localized into the membrane.

Although we did not examine interaction between DnaK and SigI or RsgI, free SigI protein in the *rsgI*::*em dnaK*::*sp* double disruptant strain BSU22 requires heat to become active. DnaK facilitates activation of heat-shocked SigI protein. Prolonged heat activates SigI in the absence of DnaK. During sporulation, free SigI protein in the *rsgI*::*em dnaK*::*sp* double disruptant strain became active without heat.

The expression or activity of BgaB may be altered by heat stress through translational or post-translational processes. However, BgaB activity from the *bgaB* gene fused to three promoters (*sigW*, *divIB* or *thiCK*) that do not respond to heat shock was stimulated only by a factor of 1.6. Introduction of *dnaK* disruption altered this factor to 1.3. Therefore the activity of BgaB was not stimulated strongly by heat, and DnaK did not seem to be required in the activity of BgaB at 50 °C. The heat induction and DnaK-dependence of BgaB activity derived from the *bgaB* gene fused to the *sigI* promoter described in the text was considered to reflect *sigI-rsgI* expression.

When a primer extension experiment was performed with a primer (5'-GATAGGGATTGCGCAAAGGAG-3') hybridizable to the original *sigI* promoter region, there were two weak but significant bands which were different from the unique band shown in Fig. 3. They seemed to indicate two transcription initiation sites at T or A residues located two and one bases upstream of the unique start site described in the text. They were heat-shock- and SigI-dependent. Even in this experiment, we did not obtain the results indicating transcription initiation sites described elsewhere (Zuber *et al.*, 2001).

In the primer extension experiment with a primer hybridizable to *bgaB*, there was another transcript (not shown in Fig. 3) from the A residue 23 bases upstream of the *sigI* initiation codon GTG. It was SigI-independent and heat (50 °C) sensitive. On the other hand, as shown in Fig. 5, in the *sigI*-disrupted strain BSU16, wild-type promoter fusion showed residual activity at 50 °C. A truncated promoter fusion with only the -10 region showed similar residual activity, and a promoterless fusion showed complete loss of BgaB at 50 °C. We did not analyse further whether the above transcript might be related to the *sigI* promoter. Nor did we further study the possibility that some sigma factor other than SigI might be involved in the residual BgaB activity described above.

Expression of the *sigI-rsgI* operon appears to be regulated at least at the transcriptional level. Initiation of transcription depended on the presence of active SigI. Transcription was strongly suppressed by glucose addition, but the presumed CcpA target-like site in the promoter region was not involved. The glucose effect was observed even in the absence of RsgI. The glucose effect described above explains the previous observation that *sigI* transcription is not observed in a minimal-glucose medium (Zuber *et al.*, 2001). Operon transcription was transiently induced at near the end of growth in a rich complex medium independent of DnaK function, and was gradually induced 1 h after the end of growth in a complex medium which supports efficient sporulation. As the *spo0A* gene was not involved, *sigI* induction was not related to sporulation, and nutrients such as glucose seemed to suppress *sigI-rsgI* transcription.

Summarizing the above results, a model of the activation process of the SigI protein is shown in Fig. 9. The N-terminal domain (~58 aa) of the transmembrane anti-sigma protein RsgI interacts directly with the SigI protein and keeps it inactive. Upon prolonged heat exposure, or a strong transient heat shock, SigI protein is released from RsgI and activated through heat- and DnaK-mediated process before integrating into the SigI-RNA polymerase core complex. Inactivation of the *rsgI* gene releases free but inactive SigI protein which is easily activated by transient heat shock in a DnaK-dependent fashion. Heat of long duration, however, activates free but inactive SigI independently of DnaK. Transient or prolonged heat may release SigI protein from some interacting negative factor(s).

Fig. 9. A model of SigI activation. The N-terminal domain of the transmembrane anti-sigma protein RsgI interacts directly with SigI protein. Upon exposure to heat, SigI protein (σ^I) is released from RsgI, and activated by heat through a DnaK-mediated rapid process or a DnaK-independent slow process before integration into the SigI-RNA polymerase core complex. SigI protein is shown as a square or a circle representing inactive or active form, respectively. E represents RNA polymerase core enzyme. See text for details.

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REFERENCES

- Asai, K., Kawamura, F., Yoshikawa, H. & Takahashi, H. (1995). Expression of *kinA* and accumulation of σ^H at the onset of sporulation in *Bacillus subtilis*. *J Bacteriol* **177**, 6679–6683.
- Asai, K., Yamaguchi, H., Kang, C.-M., Yoshida, K., Fujita, Y. & Sadaie, Y. (2003). DNA microarray analysis of *Bacillus subtilis* sigma factors of extracytoplasmic function family. *FEMS Microbiol Lett* **220**, 155–160.
- De La Penas, A., Connolly, L. & Gross, C. A. (1997). Sigma E is an essential sigma factor in *Escherichia coli*. *J Bacteriol* **179**, 6862–6864.
- Deutscher, J., Galinier, A. & Martin-Verstraete, I. (2002). Carbohydrate uptake and metabolism, pp. 129–150. In *Bacillus subtilis and its Closest Relatives: from Genes to Cells*. Edited by A. L. Sonenshein, J. A. Hoch & R. Losick. Washington, DC: American Society for Microbiology.
- Helmann, J. D. (2002). The extracytoplasmic function (ECF) sigma factors. *Adv Microb Physiol* **46**, 47–110.
- Hirata, H., Fukazawa, T., Negoro, S. & Okada, H. (1986). Structure of a β -galactosidase gene of *Bacillus stearothermophilus*. *J Bacteriol* **166**, 722–727.

- Homuth, G., Mogk, A. & Schumann, W. (1999). Post-transcriptional regulation of the *Bacillus subtilis* *dnaK* operon. *Mol Microbiol* **32**, 1183–1197.
- Hughes, K. T. & Mathee, K. (1998). The anti-sigma factors. *Annu Rev Microbiol* **52**, 231–286.
- Kunst, F. N., Ogasawara, I., Moszer, I., Albertini, A. M., Alloni, G., Azevedo, V., Bertero, M. G. Bessieres, P., Bolotin, A. & other authors (1997). The complete genome sequence of the Gram-positive bacterium *Bacillus subtilis*. *Nature* **390**, 249–256.
- Lindow, J. C., Kuwano, M., Moriya, S. & Grossman, A. D. (2002). Subcellular localization of the *Bacillus subtilis* structural maintenance of chromosomes (SMC) protein. *Mol Microbiol* **46**, 997–1009.
- Lonetto, M. A., Brown, K. L., Rudd, K. E. & Buttner, M. J. (1994). Analysis of the *Streptomyces coelicolor* *sigE* gene reveals the existence of a subfamily of eubacterial σ factors involved in the regulation of extracytoplasmic functions. *Proc Natl Acad Sci U S A* **91**, 7573–7577.
- Missiakas, D. & Raina, S. (1998). The extracytoplasmic function sigma factors: role and regulation. *Mol Microbiol* **28**, 1059–1066.
- Mittenhuber, G. (2002). An inventory of genes encoding RNA polymerase sigma factors in 31 completely sequenced eubacterial genomes. *J Mol Microbiol Biotechnol* **4**, 77–91.
- Miwa, Y., Nakata, A., Ogiwara, A., Yamamoto, M. & Fujita, Y. (2000). Evaluation and characterization of catabolite-responsive elements (*cre*) of *Bacillus subtilis*. *Nucleic Acids Res* **28**, 1206–1210.
- Morita, M. T., Tanaka, Y., Komada, T. S., Kyougoku, Y., Yanagi, H. & Yura, T. (1999). Translational induction of heat shock transcription factor sigma 32: evidence for a built-in RNA thermosensor. *Genes Dev* **13**, 655–665.
- Ohshima, H., Matsuoka, S., Asai, K. & Sadaie, Y. (2002). Molecular organization of intrinsic restriction and modification genes *BsuM* of *Bacillus subtilis* Marburg. *J Bacteriol* **184**, 381–389.
- Peterson, A., Brigulia, M., Hass, S., Hoheisel, J. D., Volker, U. & Hecker, M. (2001). Global analysis of the general stress response of *Bacillus subtilis*. *J Bacteriol* **183**, 5617–5631.
- Platt, T., Muller-Hill, B. & Miller, J. H. (1972). Assays of the *lac* operon enzymes. In *Experiments in Molecular Genetics*, pp. 352–355. Edited by J. H. Miller. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Price, C. (2002). General stress response, pp. 369–384. In *Bacillus subtilis and its Closest Relatives: from Genes to Cells*. Edited by A. L. Sonenshein, J. A. Hoch & R. Losick. Washington, DC: American Society for Microbiology.
- Price, C. W., Fawcett, P., Ceremonie, H., Su, N., Murphy, C. K. & Youngman, P. (2001). Genome-wide analysis of the general stress response in *Bacillus subtilis*. *Mol Microbiol* **41**, 757–774.
- Raina, S., Missiakas, D. & Georgopoulos, C. (1995). The *rpo* gene encoding the σ^E (σ^{24}) heat shock sigma factor of *Escherichia coli*. *EMBO J* **14**, 1043–1055.
- Raivio, T. L. & Silhavy, T. J. (2001). Periplasmic stress and ECF sigma factors. *Annu Rev Microbiol* **55**, 591–624.
- Rouviere, P. E., De Las Penas, A., Lu, C. Z., Rudd, K. E. & Gross, C. A. (1995). *rpoE*, the gene encoding the second heat-shock sigma factor, σ^E , in *Escherichia coli*. *EMBO J* **14**, 1032–1042.
- Sadaie, Y. (1998). *secA341* mutation inhibition of expression of the *Bacillus subtilis* protease gene, *aprE*. *Biosci Biotechnol Biochem* **62**, 1784–1787.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Schaeffer, P., Millet, J. & Aubert, J. P. (1965). Catabolite repression of bacterial sporulation. *Proc Natl Acad Sci U S A* **54**, 704–711.
- Stragier, P. & Losick, R. (1996). Molecular genetics of sporulation in *Bacillus subtilis*. *Annu Rev Genet* **30**, 297–341.
- Takamatsu, H., Kodama, T., Imamura, A., Asai, K., Kobayashi, K., Nakayama, T., Ogasawara, N. & Watabe, K. (2000). The *Bacillus subtilis* *yabG* gene is transcribed by SigK RNA polymerase during sporulation, and *yabG* mutant spores have altered coat protein composition. *J Bacteriol* **182**, 1883–1888.
- Vagner, V., Dervyn, E. & Ehrlich, S. D. (1998). A vector for systematic gene inactivation in *Bacillus subtilis*. *Microbiology* **144**, 3097–3104.
- Weber, H., Polen, T., Heuveling, J., Wendisch, V. F. & Hengge, R. (2005). Genome-wide analysis of the general stress response network in *Escherichia coli*: σ^S -dependent genes, promoters, and sigma factor selectivity. *J Bacteriol* **187**, 1591–1603.
- Wiegert, T., Homuth, G., Versteeg, S. & Schumann, W. (2001). Alkaline shock induces the *Bacillus subtilis* σ^W regulon. *Mol Microbiol* **41**, 59–71.
- Wösten, M. M. S. M. (1998). Eubacterial sigma factors. *FEMS Microbiol Rev* **22**, 127–150.
- Yoshimura, M., Asai, K., Sadaie, Y. & Yoshikawa, H. (2004). Interaction of *Bacillus subtilis* extracytoplasmic function (ECF) sigma factors with the N-terminal regions of their potential anti-sigma factors. *Microbiology* **150**, 591–599.
- Yuan, G. & Wong, S. L. (1995). Regulation of *groE* expression in *Bacillus subtilis*: the involvement of the σ^A -like promoter and the roles of the inverted repeat sequence (CIRCE). *J Bacteriol* **177**, 5427–5433.
- Zuber, U., Drzewiecki, K. & Hecker, M. (2001). Putative sigma factor SigI (YkoZ) of *Bacillus subtilis* is induced by heat shock. *J Bacteriol* **183**, 1472–1475.

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