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 ykrI). 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.

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) sigma 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 ykrI) (Kunst et al., 1997), which encodes a σ^H-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 \emph{B. subtilis} by constructing a strain carrying at the \emph{amyE} locus the \emph{bgaB} gene from \emph{Bacillus stearothermophilus} encoding a heat-stable \(\beta\)-galactosidase fused to the promoter of the \emph{sigI} operon (Hirata \emph{et al.}, 1986; Yuan \& Wong, 1995). Expression of \emph{bgaB} and amounts of transcript of the \emph{sigI} operon were examined in mutant strains with disrupted \emph{sigI} or \emph{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 \emph{B. subtilis} or \emph{E. coli} were grown in Luria–Bertani broth or in a complex sporulation medium (Sambrook \emph{et al.}, 1989; Schaeffer \emph{et al.}, 1965). DNA manipulation, PCR, reagents and enzymes were as described elsewhere (Miwa \emph{et al.}, 2000; Ohshima \emph{et al.}, 2002).

**Table 1.** Bacterial strains and plasmids

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<td>H. Yoshikawa*</td>
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<tr>
<td>pEr::Sp</td>
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**Construction of the \emph{B. subtilis} sigI operon.** A portion of the promoter region of the \emph{sigI} gene was synthesized by PCR using template DNA from a wild-type \emph{B. subtilis} strain and the primer pair 5'-GAAGAATTCTGGGGTGTTCTTAGCAG-3'/5'-GGAGGATCCACTGGTTTACAATTATA-3'. DNA was digested with EcoRI and BamHI, and cloned into plasmid pDLd, which carries the heat-stable \emph{bgaB} gene of \emph{B. stearothermophilus} integrated into the \emph{amyE} locus on the plasmid (Hirata \emph{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 \emph{amyE} locus the \emph{bgaB} gene with the promoter of the \emph{sigI} operon in the plasmid pDLd backbone. The resulting gene fusion was designated \emph{amyE}:: \emph{P}_{ugf}-\emph{bgaB}.

**BgaB activity.** Heat stable \(\beta\)-galactosidase activity of the \emph{bgaB} gene from \emph{B. stearothermophilus} was measured at 62 °C as described elsewhere (Yuan \& Wong, 1995; Platt \emph{et al.}, 1972; Sadaie, 1998).
Samples were heated at 68 °C for 5 min before measuring BgaB activity, to inactivate the endogenous β-galactosidase of the lacZ gene of E. coli in the integrated pMutin plasmid.

**Gene disruption.** Disruption of sigl and rsgl 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'-AGAGGATCTCCAGTGGCCTT-AGCCTTTG-3'/5'-GGAGGATCCACCTTTGGCGCCCTGGG-3' for sigl and 5'-AGAAGGGTCCACCTTTGGCGCCCTGGG-3'/5'-GGAGGATCTCCAGTGGCCTTGGG-3' for rsgl. 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 DNA disruption, we used two primer pairs, 5'-TCTTGCGGTTCACCTTTGGG-3'/5'-GGACCTAAAGAGGATTCTTGGG-3' for sigl and 5'-GGACCTAAAGAGGATTCTTGGG-3'/5'-TTGCGGCTTCCATGTTTGT-3' for rsgl. The primer extension reaction was performed with template DNA from a wild-type strain and primer pairs described below. Underlined portions of the primers possess the same sequence (as the 5' or 3' portion of the em gene of pMutin, which was amplified using pMutin DNA and the primer pair 5'-CTTAAAGAGGATTCTTGGG-3'/5'-TTGCGGCTTCCATGTTTGT-3'). The primer extension products were cloned into pGADT7. Sequencing of the downstream sigl and rsgl operon was performed as described elsewhere (Yoshimura et al., 2004). Plasmid vectors pGADT7 and pGADT7 carrying cloned B. subtilis sigl and rsgl genes were used to transform plasmid DNA from a wild-type strain and the following primer pairs: 5'-GGAGGAATCTGAAACCCGATTTAGCGAATAGGAGAATTCCATGAGGAGAAGGAACTGAC-3'/5'-gaagagaatctgaaacccgatttagggaggaattccatgaggagaaggaaactgac-3' for sigl, 5'-GGAACGATCAGAATAGGAGAATTCCATGAGGAGAAGGAACTGAC-3'/5'-GGAGGAATCTGAAACCCGATTTAGCGAATAGGAGAATTCCATGAGGAGAAGGAACTGAC-3' for rsgl. This was performed as described elsewhere (Ohshima et al., 2002).

**Cloning of sigl, rsgl and sigA into pGADT7 and pGPTK for yeast two-hybrid analysis.** Yeast two-hybrid analysis was performed as described elsewhere (Ohshima et al., 2004). Plasmid vectors pGADT7 and pGPT7 carrying cloned B. subtilis sigl and rsgl genes were used to transform mating-type yeast strains P169-4A and P169-4x, respectively. The sigl, sigA and rsgl genes, the 5'-terminal portion of rsgl and the 3'-terminal portion of rsgl were cloned into pGADT7 or pGPTK by inserting PCR-synthesized DNA fragments. PCR was performed with template DNA from a wild-type strain and the following primer pairs: 5'-GGAGGAATCTGAAACCCGATTTAGCGAATAGGAGAATTCCATGAGGAGAAGGAACTGAC-3'/5'-GGAGGATCCATGAAACCCGATTTAGCGAATAGGAGAATTCCATGAGGAGAAGGAACTGAC-3' for sigl, 5'-GGAACGATCAGAATAGGAGAATTCCATGAGGAGAAGGAACTGAC-3'/5'-GGAGGATCCATGAAACCCGATTTAGCGAATAGGAGAATTCCATGAGGAGAAGGAACTGAC-3' for rsgl, 5'-GGAACGATCAGAATAGGAGAATTCCATGAGGAGAAGGAACTGAC-3'/5'-GGAACGATCAGAATAGGAGAATTCCATGAGGAGAAGGAACTGAC-3' for sigA.

**RESULTS**

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

Northern blot analysis of the sigl promoter region (see Methods). Cells of B. subtilis strains carrying the P_sigl-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 sigl gene by em displacement (rsgl::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 sigl gene depends on Sigl.** The induction of BgaB activity by transient heat shock was confirmed in the rsgl-insertionally disrupted strain BSU16.
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 sigI-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<sub>sigI-bgaB</sub> 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 rsgI double disruptant strain BSU19 (sigI::pMutinT3) and the sigI rsgI::pMutinT3
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 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.

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**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.

**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.

**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).
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.

**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<sub>sigI</sub>-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
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 disruptive 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 (sigI::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 (sigI::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 (sigI::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. 7. DnaK-dependent activation of heat-shock-induced sigI-rsgI operon transcription.](image)
DISCUSSION

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

The predicted Rsgl 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 Rsgl interacted with Sigl. Deletion or pMutin-insertional inactivation of the *rsgl* gene resulted in effects on heat-inducible Sigl activation. The latter insertion left the N-terminal sequence including the transmembrane sequence intact. Therefore, even if Sigl-interacting sequences and the transmembrane sequence of Rsgl were retained *in vivo*, the Sigl-interacting sequence did not function. This suggests that Rsgl requires the long C-terminal domain to negatively regulate Sigl factor, or to be properly localized into the membrane.

Although we did not examine interaction between DnaK and Sigl or Rsgl, free Sigl protein in the rsgl::em dnaK::sp double disruptant strain BSU22 requires heat to become active. DnaK facilitates activation of heat-shocked Sigl protein. Prolonged heat activates Sigl in the absence of DnaK. During sporulation, free Sigl protein in the rsgl::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 *sigl* promoter described in the text was considered to reflect *sigl-rsgl* expression.

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**Fig. 8.** Induction of the Sigl-dependent transcription of the *sigl-rsgl* 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 sigl promoter in strains BSU15 (P*sigl*-bga*B*, ○), BSU17 (P*sigl*-bga*B* rsgl::em, △), BSU22 (P*sigl*-bga*B* rsgl::em dnaK::sp, ◆) or BSU28 (P*sigl*-bga*B* rsgl::em spo0A::HB, □). ▽, Growth and BgaB activity of BSU17, cells of which were grown at 37°C in Difco sporulation medium containing 0.5% glucose.
When a primer extension experiment was performed with a primer (5’-GATAGGATTCGCAAAGGAG-3’) hybridizable to the original sigl 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 Sigl-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 sigl initiation codon GTG. It was Sigl-independent and heat (50 °C) sensitive. On the other hand, as shown in Fig. 5, in the sigl-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 sigl promoter. Nor did we further study the possibility that some sigma factor other than Sigl might be involved in the residual BgaB activity described above.

Expression of the sigl-rsgl operon appears to be regulated at least at the transcriptional level. Initiation of transcription depended on the presence of active Sigl. 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 Rsgl. The glucose effect described above explains the previous observation that sigl 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, sigl induction was not related to sporulation, and nutrients such as glucose seemed to suppress sigl-rsgl transcription.

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

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