Genetic Evidence for the Actin Homolog Gene *mreBH* and the Bacitracin Resistance Gene *bcrC* as Targets of the Alternative Sigma Factor SigI of *Bacillus subtilis*[▽]

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The *Bacillus subtilis* sigI gene, which is a member of the class VI heat shock genes of the *B. subtilis* heat shock stimulon, encodes an alternative sigma factor whose regulon is poorly defined. In this study, by using a binary vector system, we showed that *B. subtilis* SigI could drive expression of a transcriptional fusion between the sigI regulatory region from *Bacillus licheniformis*, *Bacillus* sp. strain NRRL B-14911, *B. subtilis*, or *Bacillus thuringiensis* and the *xylE* reporter gene in *B. subtilis*. The transcriptional initiation sites of these fusions in *B. subtilis* were mapped by primer extension analyses. A putative consensus promoter sequence probably recognized by the *B. subtilis* SigI was thus deduced. Using a consensus sequence-based search procedure, we found putative σ^I promoters preceding the actin homolog gene *mreBH* and the bacitracin resistance gene *bcrC* of *B. subtilis*. Overexpression of the *B. subtilis* sigI gene could specifically stimulate expression of both an *mreBH* promoter region-*bgaB* fusion and a *bcrC* promoter region-*bgaB* fusion. Expression of these two fusions at the *amyE* locus of the *B. subtilis* chromosome was heat inducible and SigI dependent as revealed by sigI gene disruption experiments. Primer extension analysis showed that the identified *mreBH* and *bcrC* transcriptional start sites were at appropriate distances from their σ^I promoter elements. This further supports the notion that SigI can directly regulate *mreBH* and *bcrC* expression. Taken together, these results strongly suggest that *mreBH* and *bcrC* are new members of the SigI regulon.

The responses of the soil bacterium *Bacillus subtilis* to various environmental stresses such as high temperature (16, 33), alkaline media (7, 13), high osmolarity (14), oxidative stress (8, 16, 27, 33), and antibiotic stress (3, 5, 6, 9, 23) involve a variety of sigma factors. Induction of expression of genes encoding these sigma factors or activation of these sigma factors can direct core RNA polymerase to coordinate differential gene expression that mediates various cellular responses to allow for survival and persistence in harsh and adverse environments.

The *B. subtilis* sigI gene encodes an alternative sigma factor of the σ^{70} family (22). Transcription of sigI is heat inducible (50 or 52°C) (37) and depends on SigI (1). Disruption of the sigI gene led to a temperature-sensitive phenotype: the sigI mutant could not grow on LB plates at 55°C and did not form colonies on supplemented minimal medium plates at 54°C. The sigI gene was initially proposed to be a member of the class IV heat shock genes of *B. subtilis* (37) but was later reassigned into class VI of the *B. subtilis* heat shock stimulon (30). The *B. subtilis* sigI gene has orthologs in other *Bacillus* species, such as *Bacillus licheniformis* ATCC 14580 (26) (GenBank accession number YP_078683), *Bacillus thuringiensis* serovar Israelensis ATCC 35646 (ZP_00741267), and *Bacillus* sp. strain NRRL B-14911 (ZP_01172170). The sigI gene and its downstream *rsgI* (formerly *ykrI*) gene are cotranscribed and constitute an operon. The *rsgI* gene encodes a putative transmembrane pro-

tein that can interact with SigI and functions as an anti- σ^I factor (1). To date, little is known about the SigI regulon. In this study, we have derived a putative consensus sequence of the σ^I promoter and demonstrate that the actin homolog gene *mreBH* (10) and the bacitracin resistance gene *bcrC* (20) are targets of SigI.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* and *B. subtilis* cells were grown in Luria-Bertani (LB) medium (28). Antibiotics were used at the following concentrations ($\mu\text{g/ml}$): ampicillin, 100 (for *E. coli*); chloramphenicol, 5; tetracycline, 10; and erythromycin, 1 (for *B. subtilis*).

Construction of plasmids. To construct the sigI-disruptive plasmid pGS1651, a 0.3-kb DNA fragment containing the internal region of sigI and flanked by HindIII and BamHI sites was amplified by PCR and cloned between HindIII and BamHI sites of the thermosensitive replicative plasmid pRN5101 (12).

Various DNA fragments containing the regulatory region plus a small portion of N terminus (or not) of the sigI gene from *B. subtilis*, *B. thuringiensis*, *B. licheniformis*, or *B. sp.* strain NRRL B-14911 were amplified by PCR and cloned individually between the EcoRI and HindIII (or BamHI) sites of pLC4 (25) to generate plasmids pGS1217, pGS1395, pGS1564, and pGS1604, respectively. The lengths of PCR-amplified DNA fragments in plasmids pGS1217, pGS1395, pGS1564, and pGS1604 are 0.32, 0.71, 0.41, and 0.42 kb, respectively.

To construct a plasmid that overproduces SigI or SigV in *B. subtilis*, 0.8-kb and 0.56-kb DNA fragments carrying the Shine-Dalgarno sequence plus the sigI and sigV coding regions, respectively, and flanked by EcoRI and HindIII sites were amplified by PCR and cloned between EcoRI and HindIII sites of pHY300PLK (Takara Shuzo Co.) to generate plasmids pGS1671 and pGS1761, respectively. The promoter of the tetracycline resistance gene present in pHY300PLK can drive sigI and sigV expression in *B. subtilis*.

To construct an *mreBH* promoter region-*bgaB* fusion, a 0.4-kb DNA fragment was amplified by PCR (using primers 5'-GCGGAATTCGATGTACCTCAATA TATCG-3' and 5'-GCGGGATCCACTGCGACAACAGA-3') and cloned between EcoRI and BamHI sites of plasmid pDL (34) to generate plasmid pGS1722. To construct a *bcrC* promoter region-*bgaB* fusion containing only the

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Description ^a	Reference or source ^b
<i>E. coli</i> DH5 α	F ⁻ ϕ 80dlacZ Δ M15 Δ (lacZYA-argF) <i>recA1 gyrA endA1 relA1 supE44 hsdR17</i>	Laboratory stock
<i>B. subtilis</i> strains		
168	<i>trpC2</i>	Laboratory stock
BM1140	<i>trpC2 amyE::P_{mreBH}-bgaB</i>	This study
BM1168	<i>trpC2 amyE::P_{bcrc}-bgaB</i>	This study
BM1199	<i>trpC2 amyE::P_{mreBH}-bgaB sigI::pGS1651</i>	This study
BM1201	<i>trpC2 amyE::P_{bcrc}-bgaB sigI::pGS1651</i>	This study
<i>B. licheniformis</i> ATCC 14580		ATCC
<i>B. thuringiensis</i> serovar Israelensis ATCC 35646		ATCC
<i>Bacillus</i> sp. strain NRRL B-14911		ARSCC
Plasmids		
pDL	Integrative vector for generation of transcriptional fusions to <i>bgaB</i> ; Ap ^r Cm ^r	34
pHY300PLK	Shuttle vector; Ap ^r Tc ^r	Takara
pLC4	Promoter probe vector with <i>xylE</i> as the reporter gene; Ap ^r Cm ^r	25
pRN5101	Vector used for gene disruption; Ap ^r Em ^r	12
pGS1217	pLC4 carrying the promoter region of <i>B. subtilis sigI</i>	This study
pGS1395	pLC4 carrying the promoter region and the N-terminal region of <i>B. thuringiensis sigI</i>	This study
pGS1564	pLC4 carrying the promoter region and the N-terminal region of <i>B. licheniformis sigI</i>	This study
pGS1604	pLC4 carrying the promoter region and the N-terminal region of <i>Bacillus</i> sp. strain NRRL B-14911 <i>sigI</i>	This study
pGS1651	pRN5101 carrying an internal region of the <i>B. subtilis sigI</i> gene	This study
pGS1671	pHY300PLK carrying the <i>B. subtilis sigI</i> gene	This study
pGS1722	pDL carrying the promoter region and the N-terminal region of <i>B. subtilis mreBH</i>	This study
pGS1740	pDL carrying the σ^I promoter region of <i>B. subtilis bcrC</i> (not including the σ^X , σ^M , and σ^V promoters)	This study
pGS1761	pHY300PLK carrying the <i>B. subtilis sigV</i> gene	This study

^a Ap^r, ampicillin resistant; Cm^r, chloramphenicol resistant; Em^r, erythromycin resistant; Tc^r, tetracycline resistant.

^b ATCC, American Type Culture Collection; ARSCC, Agricultural Research Service Culture Collection.

σ^I promoter (and not the σ^X , σ^M , and σ^V promoters) of *bcrC*, a 0.25-kb DNA fragment was amplified by PCR (using primers 5'-GCGGGATCCTGTTGATC AAGTGACA-3' and 5'-GCAGGTACCAGATTGTCTGAAATAACGG-3') and cloned between BamHI and KpnI sites of plasmid pDL to generate plasmid pGS1740.

Construction of strains with a *bgaB* transcriptional fusion integrated at the *amyE* locus. pDL-derived integrative plasmids pGS1722 and pGS1740 were individually introduced into *B. subtilis* cells by protoplast transformation. Chloramphenicol-resistant candidate integrants were spotted onto LB plates containing 0.2% soluble starch and screened by staining with iodine solution (0.5% I₂, 1% KI) as previously described (21) for a lack of halos due to loss of amylase activity. This could confirm that there was correct double-crossover integration at the *amyE* locus and generated strains BM1140 and BM1168, respectively.

Disruption of the chromosomal *sigI* gene. Disruption of the chromosomal *sigI* gene of *B. subtilis* by integration of the thermosensitive replicative plasmid pRN5101-derived pGS1651 through a single crossover event was performed as previously described (12). *B. subtilis* BM1199 and BM1201 were constructed by introducing plasmid pGS1651 into BM1140 and BM1168, respectively, by the method of protoplast transformation. Transformants were first grown at the permissive temperature of 30°C and then transferred to the nonpermissive temperature of 39°C. Finally, integrants were selected on LB agar plates at 39°C for resistance to erythromycin. The correctness of integrants was verified by PCR.

RNA extraction and primer extension analysis. Total RNA was extracted from *B. subtilis* cells grown in LB medium to an absorbance at 600 nm of 0.5 by a previously described method (36). The transcriptional start site was determined by primer extension as previously described (17) using synthetic oligonucleotides 5'-ACTACTCATTAATGATAGCT-3' for *B. subtilis sigI*, 5'-ATAGCTAATTT GATAGGGAT-3' for *B. licheniformis sigI*, 5'-CCCACTTAATCTAGTAAAT T-3' for *B. thuringiensis sigI*, 5'-CACCTCTCCACCAGCAC-3' for *Bacillus* sp. strain NRRL B-14911 *sigI*, 5'-GTTCAAATAATCACCTTTTAC-3' for *B. subtilis bcrC*, and 5'-GCTGTTCCCTAAGTCAATC-3' for *B. subtilis mreBH*.

β -Galactosidase activity assay. Activity of the thermostable β -galactosidase BgaB was measured as previously described (29) but with some modifications. A portion (at least 2 ml) of the bacterial culture was pelleted and resuspended in an equivalent volume of chilled BgaB buffer (25 mM potassium phosphate [pH 6.4], 50 mM KCl, and 1 mM MgSO₄). The absorbance of the resuspended cells was measured at 600 nm. For cell permeabilization, 50 μ l of 0.1% sodium dodecyl sulfate and 100 μ l of chloroform were added to 1 ml of resuspended cells. Permeabilization was achieved by vortexing. The reaction was then initiated by adding 0.2 ml of *o*-nitrophenyl- β -D-galactopyranoside at a concentration of 4 mg per ml. After incubation at 55°C for 30 min, 0.5 ml of 1 M Na₂CO₃ was added to stop the reaction. The reaction mixture was centrifuged to remove cell debris and chloroform. Absorbance of the supernatant was recorded at 420 nm and 550 nm. BgaB activity is given in Miller units (19).

Other methods. Genomic DNAs from a variety of *Bacillus* species were isolated as previously described (24). Transformation of *B. subtilis* cells by the protoplast method was carried out as previously described (11). Assays of catechol 2,3-dioxygenase (XylE) activity were performed according to an established method (25). Protein concentrations were determined by the bicinchoninic acid protein assay method according to the instructions of the manufacturer (Pierce Biotechnology, Inc.) with bovine serum albumin as the standard.

RESULTS

Effects of overexpression of *B. subtilis sigI* on expression of transcriptional fusions of *sigI* regulatory regions from various *Bacillus* species to an *xylE* reporter gene in *B. subtilis*. In an attempt to define a consensus sequence for the σ^I promoter and to identify target genes for the sigma factor SigI of *B. subtilis*, we adopted a different approach. As mentioned above,

TABLE 2. Effects of overexpression of *B. subtilis sigI* on expression of various promoter-*xylE* transcriptional fusions in a binary-vector system of *B. subtilis*

Plasmid 1 ^a	Plasmid 2	XylE sp act (mU/mg) ^b
pGS1671 (<i>B. subtilis sigI</i> -expressing plasmid)	pGS1217 (carrying <i>B. subtilis sigI</i> promoter region- <i>xylE</i> fusion)	292.1 ± 23.7
pHY300PLK (control vector)	pGS1217	144.6 ± 15.1
pGS1671	pGS1564 (carrying <i>B. licheniformis sigI</i> promoter region- <i>xylE</i> fusion)	249.4 ± 24.6
pHY300PLK	pGS1564	93.5 ± 8.2
pGS1671	pGS1395 (carrying <i>B. thuringiensis sigI</i> promoter region- <i>xylE</i> fusion)	157.3 ± 16.9
pHY300PLK	pGS1395	26.8 ± 4.5
pGS1671	pGS1604 (carrying <i>Bacillus</i> sp. strain NRRL B-14911 <i>sigI</i> promoter region- <i>xylE</i> fusion)	488.1 ± 47.6
pHY300PLK	pGS1604	91.2 ± 9.4

^a *sigI* was overexpressed from the pHY300PLK-based plasmid pGS1671.

^b The specific activity of XylE was measured with crude extracts prepared from *B. subtilis* cells carrying a binary-vector system and grown at 37°C to an absorbance at 600 nm of 1.0. Each value is the mean ± standard error from three independent experiments.

the *B. subtilis sigI* gene and its downstream *rsgI* gene, which encodes an anti- σ^I factor, are cotranscribed and constitute an operon. Expression of the *sigI* operon is subject to positive autoregulation by SigI (1). Since a similar genetic organization of the *sigI* operon exists in *B. licheniformis* ATCC 14580, *B. thuringiensis* serovar Israelensis ATCC 35646, and *Bacillus* sp. strain NRRL B-14911, it is possible that a positive autoregulatory mechanism for expression of the *sigI* operons is also conserved in these *Bacillus* species. Since overall amino acid sequence similarities and identities between the *B. subtilis* SigI and SigI from the other three *Bacillus* species range from 94 to 70% and 83 to 48%, respectively, it is possible that the σ^I promoter sequence that can be recognized by SigI from these *Bacillus* species is also conserved.

To examine whether the *B. subtilis* SigI could recognize and activate the σ^I promoters of *sigI* genes from various *Bacillus* species, we used a binary-vector system in *B. subtilis*. In this system, one vector (plasmid pGS1671) constitutively produced the *B. subtilis* SigI and the other vector carried the transcriptional fusion of the regulatory region of *sigI* from one of these *Bacillus* species to the *xylE* reporter gene. As shown in Table 2, the specific activity of XylE from each of the *B. subtilis* strains carrying the *sigI*-overexpressing plasmid was significantly higher than that observed in the corresponding *B. subtilis*

strains carrying the control vector pHY300PLK. In a control experiment, a DNA fragment showing no promoter activity was fused to *xylE*. The result revealed that *sigI* overexpression had no effect on XylE activity in this *B. subtilis* strain (data not shown). Taken together, these results suggest that the *B. subtilis* SigI can specifically recognize and activate the σ^I promoters of *sigI* genes from these *Bacillus* species.

Mapping of transcriptional initiation sites of σ^I promoters of *sigI* genes from various *Bacillus* species in *B. subtilis*. We next used various synthetic oligonucleotides as primers to map transcriptional initiation sites of σ^I promoters of *sigI* genes from various *Bacillus* species by primer extension analyses. RNAs were isolated from *B. subtilis* cells harboring the above-mentioned binary vectors. As shown in Fig. 1, a major SigI-responsive extension product could be detected for each *sigI* gene from these *Bacillus* species. The transcriptional initiation site of the σ^I promoter of each *sigI* gene could thus be located by comparison with each corresponding DNA sequencing ladder (Fig. 1 and Table 3).

Alignment of putative σ^I promoter sequences of *sigI* genes from various *Bacillus* species. We next made an alignment of the nucleotide sequences upstream of these identified transcriptional initiation sites in order to deduce a putative consensus sequence for the σ^I promoter. Since SigI is an alterna-

FIG. 1. Primer extension analysis of transcriptional initiation sites of σ^I promoters of *sigI* from various *Bacillus* species in *B. subtilis*. (A) *B. subtilis sigI*. (B) *B. licheniformis sigI*. (C) *B. thuringiensis sigI*. (D) *Bacillus* sp. strain NRRL B-14911 *sigI*. Total RNA was isolated from *B. subtilis* cells carrying binary vectors. Lanes 1, extension product for *B. subtilis* cells carrying the control vector pHY300PLK plus a plasmid containing a *sigI* promoter region-*xylE* fusion; lanes 2, extension product for *B. subtilis* cells carrying the *sigI*-overexpressing plasmid pGS1671 plus a plasmid containing a *sigI* promoter region-*xylE* fusion. Dideoxy sequencing ladders obtained with the same primer used for each primer extension analysis are shown in the first four lanes (G, A, T, and C) of each panel. The sequences shown are complementary to those read from ladders. Each arrow indicates a transcriptional initiation site.

TABLE 3. Alignment of SigI-dependent promoters

Gene (species)	Promoter region ^a	5' Untranslated region (nucleotides)
<i>sigI</i> (<i>B. subtilis</i>)	acgcataaa ACCCCC ttAAttcttttagaaaggca CGAA atcatgtata G aacg	116
<i>sigI</i> (<i>B. licheniformis</i>)	ggcggggaaa ACCCCC ttAAtcgtcaaacagatca CGAA ttgttctagg A gatc	115
<i>sigI</i> (<i>B. thuringiensis</i>)	tattttttt ACCCCC atAAaactatgtattcttc CGAA tatgtatagt G aaga	376
<i>sigI</i> (<i>Bacillus</i> sp. strain NRRL B-14911)	caccaaacc ACCCCC aa AA gctcctcttttcggg CGAA gctttatgtta G cag	95
<i>bcrC</i> (<i>B. subtilis</i>)	gcacagaat CCCCC ag AA accgcgattctctt CGAA ttctcttca A gcgc	92
<i>mreBH</i> (<i>B. subtilis</i>)	cgcacgcac ACCCCC aa AA atcgcatgtattctt AGAA actttaaatg T agaa	39
Consensus	----- ACCCCC --- AA ----- CGAA -----	

^a The -35 region, two consecutive A nucleotides downstream of the -35 region, and the -10 region (from left to right) are indicated in bold uppercase letters. The identified transcriptional initiation sites (+1) are denoted by underlined bold uppercase letters.

tive sigma factor of the σ^{70} family, σ^I promoters are thought to contain -35 and -10 consensus elements (22). As shown in Table 3, the putative consensus sequence for the σ^I promoter is ACCCCC for the -35 region and CGAA for the -10 region. The spacing between the -35 and -10 regions is 19 nucleotides. It is interesting to note that there is a conserved sequence "AA" within the spacer and close to the -35 region.

Computer search for *B. subtilis* genes preceded by putative σ^I promoter elements. Identification of target genes in the *B. subtilis* genome by using a consensus sequence-directed computer search has been reported for the *B. subtilis* sigma factor SigX (15). In this study, we used the consensus sequence of the putative σ^I promoter elements deduced from the analyses described above to search the SubtiList database (<http://genolist.pasteur.fr/SubtiList/>) for possible SigI target genes. The search pattern we used was ACCCCC-N₂-AA-N₁₅-CGAA (N represents any base), where one mismatch was allowed in either ACCCCC or CGAA. In an initial search, the search regions were arbitrarily restricted to being within 200 bp upstream of a predicted gene. This search identified *bcrC* (formerly *ywoA*; bacitracin-resistant protein), *lytE* (cell wall hydrolase), *mreBH* (cell shape-determining protein), *ysfD* (possible glycolate oxidase subunit), and *ytpS* (possible DNA translocase) as potential target genes.

Effects of overexpression of *B. subtilis* *sigI* on expression of candidate target genes. To test whether *sigI* overexpression could stimulate expression of these candidate target genes, PCR-amplified DNA fragments containing putative σ^I promoter elements of these candidate genes were individually cloned into the promoter probe vector pDL (34), which was designed to create transcriptional fusions to *bgaB* (encoding a thermostable β -galactosidase). It was previously shown that the *bcrC* gene could be directly regulated by three extracytoplasmic function sigma factors: SigX, SigM, and SigV (4, 18, 35). Therefore, the DNA fragment used in this report did not include the σ^X , σ^M , and σ^V promoter regions so as to avoid possible interference. The generated plasmids carrying *bgaB* fusions were individually introduced into *B. subtilis* and integrated into the chromosome by a double crossover at the *amyE* locus. The pHY300PLK-based plasmid pGS1671 was then introduced into these strains to constitutively overexpress SigI. The results showed that *sigI* overexpression could stimulate expression of both the *bcrC* promoter region-*bgaB* fusion and the *mreBH* promoter region-*bgaB* fusion (Fig. 2A and B) but could not stimulate expression of a *lytE*, *ysfD*, or *ytpS* promoter

region-*bgaB* fusion at the *amyE* locus (data not shown). In a control experiment, we used *B. subtilis* strains carrying either the *bcrC* promoter region-*bgaB* fusion or the *mreBH* promoter region-*bgaB* fusion at the *amyE* locus plus the pHY300PLK-based plasmid pGS1761, which constitutively overexpressed the sigma factor SigV (32). It turned out that *sigV* overexpression could not increase BgaB activity in these strains (data not shown). Together, these results suggest that SigI can specifically stimulate *bcrC* and *mreBH* expression and that the *lytE*, *ysfD*, and *ytpS* genes are not SigI targets.

SigI-dependent heat induction of *bcrC* and *mreBH*. It was previously shown that expression of the *B. subtilis* *sigI* gene was heat inducible (37). This prompted us to test whether expression of SigI target genes could also be induced by heat. We

FIG. 2. Effects of overexpression of *B. subtilis* *sigI* on expression of *bcrC* and *mreBH* promoter region-*bgaB* transcriptional fusions. (A) Expression of a *bcrC* promoter region-*bgaB* fusion at the *amyE* locus of BM1168 carrying the *sigI*-overexpressing plasmid pGS1671 (filled circles) or the control vector pHY300PLK (filled squares). (B) Expression of an *mreBH* promoter region-*bgaB* fusion at the *amyE* locus of BM1140 carrying the *sigI*-overexpressing plasmid pGS1671 (filled circles) or the control vector pHY300PLK (filled squares). Cells were grown in LB medium for various times as indicated. Growth was monitored by measuring optical density at 600 nm (OD₆₀₀) and is indicated by open symbols. The values shown are means from two independent experiments. Individual values did not differ by more than 15% from the means.

FIG. 3. SigI-dependent heat induction of a *bcrC* promoter region-*bgaB* fusion and an *mreBH* promoter region-*bgaB* fusion. Cells were grown at 37°C to an absorbance at 600 nm of 0.3 and then divided into two parts. One part remained at 37°C, and the other was transferred to 51°C at time zero. The cells were grown for various times as indicated. (A) BgaB activity from the *bcrC* promoter region-*bgaB* fusion was measured in the wild-type strain BM1168 at 37°C (open circles) or 51°C (filled circles) or in the *sigI*-disrupted strain BM1201 at 37°C (open triangles) or 51°C (filled triangles). (B) BgaB activity from the *mreBH* promoter region-*bgaB* fusion was measured in the wild-type strain BM1140 at 37°C (open circles) or 51°C (filled circles) or in the *sigI*-disrupted strain BM1199 at 37°C (open triangles) or 51°C (filled triangles). Growth was monitored by measuring optical density at 600 nm (OD₆₀₀) and is indicated by dashed lines. The values shown are means from two independent experiments. Individual values did not differ by more than 15% from the means.

used the chromosomal *bgaB* transcriptional fusions at the *amyE* locus as described above to evaluate the heat inducibility of these target genes. As shown in Fig. 3A and B, both *bcrC* and *mreBH* expression could be induced by heat. To investigate whether heat induction of *bcrC* and *mreBH* was SigI dependent, we disrupted the chromosomal *sigI* gene in these strains. The result showed that *sigI* disruption almost totally abolished heat induction of the *bcrC* promoter region-*bgaB* fusion and the *mreBH* promoter region-*bgaB* fusion (Fig. 3A and 3B). This indicates that heat induction of *bcrC* and *mreBH* can be SigI dependent.

Mapping of transcriptional initiation sites of σ^I promoters of the *bcrC* and *mreBH* genes. To further demonstrate that SigI could regulate *bcrC* and *mreBH* expression directly, we performed primer extension analysis to determine the transcriptional start sites of σ^I promoters of the *bcrC* and *mreBH* genes. RNAs were isolated from *B. subtilis* cells carrying a *bcrC* promoter region-*bgaB* fusion or *mreBH* promoter region-*bgaB* fusion at the *amyE* locus plus the *sigI*-overexpressing plasmid pGS1671 or the control vector pHY300PLK. Figure 4A and B show that a major SigI-responsive extension product could be detected for the *bcrC* and *mreBH* genes. The corresponding transcriptional start sites were at appropriate distances from the σ^I promoter elements of *bcrC* and *mreBH* (Table 3). This

FIG. 4. Primer extension analysis of transcriptional initiation sites of σ^I promoters of the *bcrC* and *mreBH* genes. (A) Extension product for *B. subtilis* cells carrying a *bcrC* regulatory region-*bgaB* fusion at the *amyE* locus plus the control vector pHY300PLK (lane 1) or the *sigI*-overexpressing plasmid pGS1671 (lane 2). (B) Extension product for *B. subtilis* cells carrying an *mreBH* promoter region-*bgaB* fusion at the *amyE* locus plus the control vector pHY300PLK (lane 1) or the *sigI*-overexpressing plasmid pGS1671 (lane 2). Dideoxy sequencing ladders obtained with the same primer used for each primer extension analysis are shown in the first four lanes (G, A, T, and C) of each panel. The sequences shown are complementary to those read from ladders. Each arrow indicates a transcriptional initiation site.

further supports the notion that SigI can directly regulate *bcrC* and *mreBH* expression.

DISCUSSION

When the first paper proposing that the *B. subtilis* *ykoZ* gene encodes a putative sigma factor, SigI, was published in 2001, the authors mentioned that DNA microarray experiments were in progress so as to define the SigI-dependent regulon (37). However, no further microarray data have been reported since then. To date, no SigI target gene except *sigI* itself has been identified (1). In this report, we have successfully used a different approach to demonstrate that the *bcrC* and *mreBH* genes are new members of SigI regulon.

The *B. subtilis* *bcrC* gene encodes an undecaprenyl pyrophosphate phosphatase that is important for cell wall biosynthesis (2) and for bacitracin resistance (20). Bacitracin is an antibiotic that binds to undecaprenyl pyrophosphate and prevents its dephosphorylation into undecaprenyl phosphate. Undecaprenyl phosphate is a lipid carrier that is required for cell wall biosynthesis (31). The *B. subtilis* *mreBH* gene encodes a bacterial homolog of actin that is important for cell morphogenesis. An *mreBH* mutant exhibits a cell wall-related defect (10). The critical role of *B. subtilis* SigI in regulation of *bcrC* and *mreBH* implies that one function of SigI is related to the maintenance of cell envelope integrity and homeostasis during heat stress. It was previously observed that a *B. subtilis* *sigI* mutant (BFA 251) exhibited a temperature-sensitive phenotype (37). The *B. subtilis* *sigI* mutant that we constructed exhibited a similar phenotype. However, this phenotype was not observed in either the *bcrC* or the *mreBH* mutant (data not shown), suggesting that another, not-yet-identified SigI-dependent gene(s) may contribute to heat resistance.

In addition to the initial computer search in which the search region was limited to 200 bp upstream of a predicted gene, we also carried out a more extensive search by not restricting the search region to 200 bp upstream of a predicted gene. We also

did additional computer searches by allowing one mismatch in the -35 region, one mismatch in the -10 region, and one or two mismatches in the highly conserved AA sequence simultaneously. Since it is unusual that there is a string of five consecutive Cs in the consensus sequence of the -35 region of the putative σ^I promoter, we selected those genes containing at least four consecutive Cs in the -35 region as potential candidate genes. By using these criteria, we were able to find more potential target genes, including the *cox4* (spore cortex protein), *dnaJ* (heat shock protein), *mbf* (MreB-like protein), *metA* (homoserine *O*-succinyltransferase), *rapK* (response regulator aspartate phosphatase), *sacX* (negative regulatory protein of SacY), and *ywjE* (possible cardiolipin synthetase) genes. We tested each of them and found that *sigI* overexpression could not stimulate their expression (data not shown).

The *B. subtilis bcrC* gene was known to be directly regulated by three extracytoplasmic function sigma factors: SigX, SigM, and SigV (4, 18, 35). It is now clear that SigI is the fourth sigma factor that can directly regulate *bcrC* expression. Involvement of multiple sigma factors in controlling *bcrC* expression allows *bcrC* to respond to various environmental stresses through different pathways. For examples, bacitracin induction of *bcrC* expression is SigM dependent but not SigX dependent (4), and heat induction of *bcrC* can be mediated through SigI, as revealed in this report.

Several attempts to carry out in vitro runoff transcription experiments by using purified His-tagged SigI and His-tagged core RNA polymerase have been unsuccessful. Since overexpression of *sigI* in *Escherichia coli* produced only insoluble SigI protein in inclusion bodies, we tried several different methods to refold denatured SigI protein, including (i) dialysis of a denatured SigI-containing solution against buffers, (ii) gradual dilution of a denatured SigI-containing solution with buffers, and (iii) on-column refolding of denatured SigI by using a descending concentration gradient of urea. Unfortunately, the SigI proteins prepared by these methods could not work in the in vitro transcription experiments. Several possibilities may explain the failure of these attempts: (i) a lack of some general or specific chaperone(s) that might be required in vivo to help folding of SigI to a biologically active form, (ii) a lack of an unidentified protein that might be required in vivo to facilitate the association of SigI with core RNA polymerase, or (iii) a lack of an unknown protein that might be required in vivo for the σ^I holoenzyme to bind specifically to its promoter DNA. Nevertheless, this question remains to be clarified.

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