Transcription of genes near the *sspE* locus of the *Bacillus subtilis* genome

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The *yfhP*, *yfhQ* (*mutY* homologue), *yfhS* and *yfhR* (oxidoreductase homologue) genes, which are located upstream of the *sspE* locus, have been identified in the *Bacillus subtilis* genome. Transcriptional analysis showed that *yfhP*, *yfhQ* and *yfhR* are transcribed during the exponential growth phase, and *sspE* is monocistronically transcribed in the late sporulation phase and co-transcribed with *yfhQ* and/or *yfhR* during exponential growth. However, *SspE* was not translated during this period. Northern blot and primer extension analyses indicated that *yfhS* is transcribed by $E\sigma^E$ during sporulation. No significant difference between wild-type and *yfhS* mutant strains was found in the rate of sporulation or germination, the heat tolerance of spores or the transcription of the *sspE* locus during sporulation. The transcription of the *yfhP* and *yfhQ*–*yfhR*–*sspE* loci increased 2.5- and 5.3-fold in a *yfhP*-deficient strain compared to the wild-type strain at t_{-2} (2 h before initiation of sporulation). In addition, transcription corresponding to the *yfhR*–*sspE* loci increased more than twofold with maximum values observed at $t_{-1.5}$. These results suggest that *YfhP* may act as a negative regulator for the transcription of *yfhQ*, *yfhR*, *sspE* and *yfhP*.

Keywords: genome analysis, *sspE* region, *MutY* homologue, oxidoreductase homologue, transcriptional regulation

INTRODUCTION

Analysis of the *Bacillus subtilis* genome sequence has revealed ORFs *yfhP*, *yfhQ* and *yfhR* upstream of the *sspE* locus (Kunst *et al.*, 1997). *sspE* encodes a small acid-soluble spore protein and is monocistronically transcribed by $E\sigma^G$ at stage III of sporulation (Hackett & Setlow, 1987; Fajardo-Cavazos *et al.*, 1991). *yfhQ* probably encodes a *B. subtilis* *mutY* counterpart since it has high similarity to the A/G-specific adenine glycosylase of *Escherichia coli* (38.0% identity over 271 residues) (Michaels *et al.*, 1990). *YfhP* appears to be a 37.1 kDa basic membrane-anchored protein without any significant similarity to known proteins.

A small ORF, designated *yfhS*, which was not referred to by Yamamoto *et al.* (1996), encodes a protein of 74 aa with a molecular mass of 8797 Da. We report here data on the transcriptional analysis of *yfhP*, *yfhQ*, *yfhS* and *yfhR*, and regulation of transcription of the *yfhP* and *yfhQ*–*yfhR*–*sspE* operons by *yfhP* during exponential growth.

METHODS

Bacterial strains, plasmids and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. Bacteria were cultured in LB medium (g l⁻¹: yeast extract, 5; tryptone, 10; NaCl, 5; pH 7.2) at 37 °C. Ampicillin or erythromycin was added to a final concentration of 50 or 0.3 µg ml⁻¹, respectively. Schaeffer medium (Schaeffer *et al.*, 1965) was used for *B. subtilis* sporulation.

Plasmid construction. Derivatives of pMUTIN1 (Vagner *et al.*, 1998) and pGEM3zf(+) (Promega) were used to construct mutants of *B. subtilis* and to prepare gene-specific RNA probes, respectively. Plasmids pMYFHQ, pMYFHS and pMSSPE were constructed by cloning *Hind*III–*Bam*HI fragments of PCR products amplified with primers HYFHQ and BYFHQ, hSHF and hSBR, and HSSPE and BSSPE into vector pMUTIN1, respectively (Table 2 and Fig. 1). These *Hind*III–*Bam*HI-digested PCR fragments were also cloned into pGEM3zf(+) to generate plasmids pGYFHQ, pGYFHS and pGSSPE, respectively. To clone the internal region of *yfhR*, the 2.4 kb fragment amplified with primers HYFHQ and BSSPE was digested with *Hind*III and *Bgl*II and the resultant 445 bp *Hind*III–*Bgl*II fragment was ligated into the *Hind*III/*Bam*HI sites of pMUTIN1 and pGEM3zf(+) to generate

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype	Source or reference*
<i>Bacillus subtilis</i>		
168	<i>trpC2</i>	D. Ehrlich
YFHPd	<i>trpC2 yfbP::pMYFHP</i>	This study
YFHQd	<i>trpC2 yfbQ::pMYFHQ</i>	This study
YFHSd	<i>trpC2 yfbS::pMYFHS</i>	This study
YFHRd	<i>trpC2 yfbR::pMYFHR</i>	This study
SSPEd	<i>trpC2 sspE::pMSSPE</i>	This study
SELZ	<i>trpC2 sspE::pMSELZ</i>	This study
AMLZ	<i>trpC2 amyE::(cat lacZ)</i>	This study
AMP2	<i>trpC2 amyE::(cat P_{yfbQ} yfbQ-lacZ)</i>	This study
AMP345	<i>trpC2 amyE::(cat P_{yfbR} yfbR-lacZ)</i>	This study
IS38	<i>trpC2 spoIIIC94</i>	BGSC
IS60	<i>leuA8 tal-1 spoIIG41</i>	BGSC
spoIIIGA1	<i>trpC2 spoIIIGA1</i>	P. Setlow
497.1	<i>trpC2 spoIIID83</i>	Kunkel <i>et al.</i> (1988)
HSIID	<i>trpC2 spoIIID83 yfbS::pMYFHS</i>	YFHSd → 497.1†
<i>Escherichia coli</i>		
JM109	<i>recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi Δ(lac-proAB)/F' [traD36 proAB' lacI^q lacZΔM15]</i>	Takara Shuzo
C600	<i>supE44 hsdR thi-1 thr-1 leuB6 lacY1 tonA21</i>	Laboratory stock
Plasmid		
pMUTIN1	<i>erm bla lacZ lacI Pspac</i>	Vagner <i>et al.</i> (1998)
pGEM3zf(+)	<i>bla lacZ</i>	Promega
pMYFHP	<i>pMUTIN1::ΔyfbP</i>	This study
pMYFHQ	<i>pMUTIN1::ΔyfbQ</i>	This study
pMYFHS	<i>pMUTIN1::ΔyfbS</i>	This study
pMYFHR	<i>pMUTIN1::ΔyfbR</i>	This study
pMSSPE	<i>pMUTIN1::ΔsspE</i>	This study
pMSELZ	<i>pMUTIN1::sspE-lacZ translational fusion</i>	This study
pGYFHP	<i>pGEM3zf(+):ΔyfbP</i>	This study
pGYFHQ	<i>pGEM3zf(+):ΔyfbQ</i>	This study
pGYFHS	<i>pGEM3zf(+):ΔyfbS</i>	This study
pGYFHR	<i>pGEM3zf(+):ΔyfbR</i>	This study
pGSSPE	<i>pGEM3zf(+):ΔsspE</i>	This study
pDH88	<i>bla cat lacI Pspac</i>	Henner (1990)
pDHAF	<i>bla cat lacI Pspac ΔamyE (front region)</i>	This study
pDHAFB	<i>bla amyE::(cat lacI Pspac)</i>	This study
pDHAFBLZ	<i>bla amyE::(cat lacZ)</i>	This study
pDHLZP2	<i>bla amyE::(cat P_{yfbQ} yfbQ-lacZ)</i>	This study
pDHLZP345	<i>bla amyE::(cat P_{yfbR} yfbR-lacZ)</i>	This study

* BGSC, Bacillus Genetic Stock Center, Ohio State University, USA; D. Ehrlich, INRA, Jouy-en-Josas, France; P. Setlow, Health Center, Farmington, CT, USA.

† *B. subtilis* 497.1 was transformed by YFHSd DNA.

Table 2. Primers used in this study

Primer	Sequence (5' → 3') or source*	Restriction enzyme
HYFHQ	GCCGAAGCTTAGACATACAGCAATTTTCGC	<i>Hind</i> III
BYFHQ	GCGCGGATCCAACAATGCCGCCGTATTC	<i>Bam</i> HI
hSHF	GCCGAAGCTTATGAGCGAATTGAACATGG	<i>Hind</i> III
hSBR	GCGGGATCCTCAATTTCTTGCCTTAATTCC	<i>Bam</i> HI
HSSPE	GCCGAAGCTTGCTAACTCAAATAACTTCAGC	<i>Hind</i> III
BSSPE	GCGGGATCCCTGTCTTACCTGCTGTGC	<i>Bam</i> HI
BYFHP	GCGCGGATCCTACCAAATCGCGATAGCTG	<i>Bam</i> HI
HFLZsE	CGGCAGCTTGAAGATCTGCTTGAGGATG	<i>Hind</i> III
SRLZsE	GCGCGTCGACCATTTGTTATCACCTCCAC	<i>Sal</i> I
AFSma-1F	TCCCCCGGGTGATTTGTATTCACTCTGCC	<i>Sma</i> I
ABXA-3R	GCTCTAGACGCTCTTTTTCGCTTGGTTGTATCC	<i>Aat</i> II
ABEco-2F	CCGGAATTCAAATGCAGGTTTCATCCTCTG	<i>Eco</i> RI
P2MUTY-1E	GCGCGAATTCAAAGACATGCAGAACGAC	<i>Eco</i> RI
P2MUTY-2S	GCCGCCCGGGTTTGTCTTCAAGTACGTTT	<i>Sma</i> I
P345HR-1E	GCGCGAATTCCCGAATCTTGAAAC	<i>Eco</i> RI
P345HR-2S	GCCGCCCGGGTGCTTCTGTTACGAGTG	<i>Sma</i> I
–21M13	Universal primer (Takara)	
M13RV	Universal primer (Takara)	
yfhP-ex1	GATCATAACAGCGTGAGC	
yfhQ-ex1	CTCTCGTTCAAACCATGAG	
yfhR-ex1	CGTGCATAGTTAATGACG	
yfhR-ex2	GTTATGTATGTCGGACGTG	
yfhR-ex3	TCTCTTACGATTAAGAATGG	
yfhS-ex1	GTTCAGTCTTCTTCCAATC	

* Additional sequences which do not correspond to the sequences near the *sspE* locus are italicized. Restriction sites for the enzymes listed in the third column are shown in bold.

Fig. 1. Physical map and gene organization of the *yfhP*, *yfhQ*, *yfhS*, *yfhR* and *sspE* loci. *yfhS* is a gene newly identified in this study. The five deduced ORFs and their translational and translational directions are indicated by arrows and arrowheads, respectively. Plasmids used as specific probes for Northern analysis are shown above the map and the thick bars indicate the regions cloned into the parent plasmid, pMUTIN1. The deduced ρ -independent terminators are denoted as follows: T1, $\Delta G = -18.4 \text{ kcal mol}^{-1}$; T2, $\Delta G = -22.7 \text{ kcal mol}^{-1}$; T3, $\Delta G = -32.8 \text{ kcal mol}^{-1}$. The hooked arrows indicate the transcriptional start points and directions, as shown in Fig. 4. H, N and B denote *Hind*III, *Not*I and *Bgl*II sites, respectively.

plasmids pMYFHR (Fig. 1) and pGYFHR, respectively. To clone the internal region of *yfhP*, the 1.0 kb fragment amplified with primers BYFHP and BYFHQ was digested with *Hind*III and *Bam*HI and the resultant 300 bp *Hind*III–*Bam*HI fragment was ligated into the *Hind*III/*Bam*HI sites of pMUTIN1 and pGEM3zf(+) to generate plasmids pMYFHP (Fig. 1) and pGYFHP, respectively. For construction of the *sspE*–*lacZ* translational fusion strain, a 229 bp PCR product with primers HFLZsE and SRLZsE was digested with *Hind*III and *Sal*I. The resulting fragment was cloned into the *Hind*III/*Sal*I sites of pMUTIN1 in-frame with the *lacZ* coding region to obtain pMSELZ.

Northern blot analysis. A culture suspension was centrifuged at various time points during growth. Each pellet ($\text{OD}_{600} = 20$) was washed in 1 ml chilled killing buffer (Volker *et al.*, 1994). After centrifugation, the pellet was resuspended in 1 ml SET buffer (Kuroda & Sekiguchi, 1993) containing 6 mg lysozyme. The suspension was kept on ice for 15 min and then centrifuged for 1 min in a microcentrifuge. The pellet was used for RNA preparation with ISOGEN (Nippon Gene) according to the manufacturer's instructions. Northern blot analysis of RNAs fractionated by electrophoresis in agarose/formaldehyde gels was performed as described by Sambrook *et al.* (1989). Probe labelling was performed with a DIG RNA Labelling Kit (Boehringer Mannheim) according to the manufacturer's instructions with some minor modifications. Briefly, the internal regions cloned into pGEM3zf(+) derivatives corresponding to individual genes were amplified by PCR with

primers —21M13 and M13RV. The amplified fragments were digested with *Hind*III and used as templates for *in vitro* run-off transcription with T7 RNA polymerase. Hybridization signals were quantified using NIH Image 1.55 software.

Primer extension analysis. Primer extension analysis was performed as described previously (Kuroda & Sekiguchi, 1993) with end-labelled primers yfhP-ex1, yfhQ-ex1, yfhR-ex1, yfhR-ex2, yfhR-ex3 and yfhS-ex1 (Table 2). The reaction products were quantified using a PhosphorImager system and ImageQuant software (Molecular Dynamics).

Construction and phenotypic characterization of *yfhP*, *yfhQ*, *yfhS*, *yfhR* and *sspE* mutants, and a *sspE-lacZ* translational fusion strain. Conventional transformation of *B. subtilis* was performed by the procedure of Anagnostopoulos & Spizizen (1961). Strains YFHPd, YFHQd, YFHSd, YFHRd, SSPEd and SELZ were constructed by integration of pMYFHP, pMYFHQ, pMYFHS, pMYFHR, pMSSPE and pMSELZ, respectively. Correct integration was confirmed by Southern hybridization. The morphology, growth rate, sporulation frequency and germination of mutants were tested.

Construction of transcriptional fusion strains between putative promoters and *lacZ* at the *amyE* locus. To obtain transcriptional fusion strains between putative promoters upstream of *yfhQ* and *yfhR*, and the *lacZ* gene, plasmid pDHAFBLZ was constructed. An *amyE* front region was amplified with primers AFSma-1F and ABXA-3R and the amplified fragment (2.05 kb) was digested with *Clal* and *Sma*I. A 0.7 kb fragment was filled in with Klenow fragment and inserted into the *Bst*1107I and *Tth*1111I sites of pDH88 to obtain pDHAF. To generate pDHAFB, an *amyE* back region was amplified with primers ABeco-2F and ABXA-3R and the amplified fragment (0.63 kb) was digested with *Aat*II and *Eco*RI. The resulting fragment was ligated with *Aat*II/*Eco*RI-digested pDHAF. Finally, to obtain pDHAFBLZ, a 3.26 kb *Eco*RI-*Dra*I fragment (containing *lacZ*) from pMUTIN1 was inserted into the *Eco*RI and *Eco*RV sites of pDHAFB. A 0.56 kb fragment containing the P2 promoter region was amplified with primers P2MUTY-1E and P2MUTY-2S and ligated into the *Eco*RI and *Sma*I sites of pDHAFBLZ to obtain pDHLZP2. A 0.65 kb fragment containing P3, P4 and P5 promoter regions was amplified with primers P345HR-1E and P345HR-2S. The amplified fragment was cloned into pDHABLZ to construct pDHLZP345 as described above. pDHLZP2, pDHLZP345 and pDHAFBLZ were linearized with *Sca*I and used for transformation of strain 168, resulting in strains AMP2, AMP345 and AMLZ, respectively. Correct integration of the plasmids into the strains was confirmed by examining halo formation on starch plates and long-range PCR.

β -Galactosidase assay. Cells were pre-cultured on Schaeffer agar plates without antibiotics at 30 °C for 10 h. Cells were then inoculated into 100 ml fresh Schaeffer medium at a density of OD₆₀₀ = 0.01 and grown at 37 °C. The β -galactosidase assay was carried out as described by Shimotsu & Henner (1986). One unit of β -galactosidase activity was defined as the amount of enzyme necessary to release 1 nmol 2-nitrophenol from ONPG in 1 min.

RESULTS AND DISCUSSION

Northern blot analysis and primer extension analysis

Figs 2–4 show the results of Northern hybridization and primer extension analysis of transcripts. The interpret-

ation of these results is summarized in Table 3. The *sspE*-specific probe was hybridized with a 0.3 kb transcript at 2.5 ($t_{2.5}$) and 5 h (t_5) after initiation of sporulation (Fig. 2e). This signal corresponds to a transcript which starts from the $E\sigma^A$ -driven promoter described by Hackett & Setlow (1987) and Fajardo-Cavazos *et al.* (1991). A weak signal corresponding to 1.8 kb was also detected (Fig. 2e). This signal does not correspond to an area covered by both *yfhR* and *sspE*, since the *yfhR*-specific probe did not hybridize with it (Fig. 2c). Therefore, it may have resulted from a read-through transcript downstream of the T3 terminator (Fig. 1). On the other hand, three transcripts estimated to be 1.3 kb (*yfhR-sspE*), 1.5 kb (*yfhR-sspE*) and 2.6 kb (*yfhQ-yfhR-sspE*) were detected during the exponential growth phase (Fig. 2c, e). *yfhQ* was transcribed as a 2.6 kb mRNA and a 1.2 kb monocistronic mRNA in the same period (Fig. 2b). Moreover, *yfhP* was monocistronically transcribed during the exponential growth phase (Fig. 2a). As shown in Fig. 2(d), the *yfhS*-specific probe hybridized with a 0.3 kb transcript at $t_{2.5}$ and t_5 . This suggests that *yfhS* is a functional ORF monocistronically transcribed during sporulation.

To determine the start points of transcripts starting between *yfhQ* and *yfhR*, primer extension was performed with oligonucleotides yfhR-ex1, yfhR-ex2 and yfhR-ex3. The results indicated that P4 (Fig. 3d) and P5 (Fig. 3e) are located in the region between the putative T2 terminator and *yfhR* (Fig. 4b), whereas P3 (Fig. 3c) is located in an upstream region encoding the C terminus of YfhQ (Fig. 4b). As revealed by Northern blotting with the *yfhQ*-specific probe (Fig. 2b), the putative T2 terminator seems to be partially read through. The —35 and —10 regions found upstream of P3 and P5 show similarity to the σ^A consensus (Table 3 and Fig. 4b). This suggests that P3 and P5 are recognized by $E\sigma^A$. On the other hand, the promoter region of P4 does not correspond to the consensus sequence of any known σ factors, including σ^A (Fig. 4b). Moreover, the transcript derived from P4 was very weak and we did not observe a corresponding signal on Northern blot analysis (Fig. 2c, e). However, a signal was observed by primer extension analysis with both yfhR-ex1 (data not shown) and yfhR-ex2 (Fig. 3d). We also tried to determine the 5' ends of transcripts corresponding to *yfhQ* or *yfhP*. A primer extension signal (P2) was obtained with primer yfhQ-ex1 and RNA from wild-type cells at t_{-2} (Fig. 3b). Assuming that mRNAs which start from P2 end at the T2 and T3 terminators, the lengths of these transcripts would be 1.17 and 2.63 kb, respectively. These data are consistent with the results of Northern blot analysis (Fig. 2b). In the case of *yfhP*, a primer extension signal (P1) was obtained with primer yfhP-ex1 and RNA from the cells at t_{-2} (Fig. 3a). The 1.1 kb signal in Fig. 2(a) seems to correspond in size to mRNA which starts from P1 and stops at T1. The —35 (TTTTCT) and —10 (TAACAT) regions spaced 16 bp apart were found upstream of P2, and the —35 (TTTTCT) and —10 (TACACT) regions spaced 18 bp apart found upstream of P1 do not seem to correspond to the σ^A consensus

Table 3. Various transcripts detected in the *yfhPQRS* and *sspE* loci

Start point	Size on Northern blot (kb)*	Corresponding locus	Expression period	– 35 region	Space	– 10 region	Sigma factor
P1	1.1	<i>yfhP</i>	Vegetative	TTTTCT	16	TACACT	?
P2	2.6	<i>yfhQ</i> – <i>yfhR</i> – <i>sspE</i>	Vegetative	TTTTCT	18	TAACAT	?
	1.2	<i>yfhQ</i>	Vegetative	–	–	–	
P3	1.5	<i>yfhR</i> – <i>sspE</i>	Vegetative	TGGAAT	17	TAAAGT	A
P4	ND	<i>yfhR</i> – <i>sspE</i>	Vegetative	–	–	–	?
P5	1.3	<i>yfhR</i> – <i>sspE</i>	Vegetative	TTGGCC	18	CATAAT	A
P6	0.3	<i>yfhS</i>	Sporulation	GCGTATA	15	CAAACAAT	E
P7†	0.3	<i>sspE</i>	Sporulation	GAATA	18	CATTCTA	G

* ND, Not determined.

† The P7-initiated transcript was previously reported by Fajardo-Cavazos *et al.* (1991). **Fig. 2.** Northern blot analysis with *yfhP*- (a), *yfhQ*- (b), *yfhR*- (c), *yfhS*- (d) and *sspE*-specific (e) RNA probes. All probes were labelled with digoxigenin as described in Methods. Each lane contained 10 µg total RNA from *B. subtilis* wild-type strain 168 at t_{-2} (lanes 1), t_{-1} (2), t_0 (3), t_1 (4), $t_{2.5}$ (5), or t_5 (6). rRNA positions are indicated (23S, 2.93 kb 23S rRNA; 16S, 1.55 kb 16S rRNA).

(Haldenwang, 1995) (Table 3 and Fig. 3a). Also the –35 regions of P2 and P1 do not seem to match any of the known promoter consensus sequences, but they are

100% identical to each other. This suggests that they are recognized by the same regulatory protein or an unknown σ factor.

Fig. 3. Determination of transcriptional start points by primer extension analysis. RNA (40 µg in panels a, b, d and f, and 20 µg in panels c and e) from *B. subtilis* wild-type strain 168 (W) at t_{-2} , t_3 or $t_{4.5}$, *B. subtilis* IS60 (σ^E ; E) at $t_{4.5}$, *B. subtilis* spoIIIG Δ 1 (σ^G ; G) at $t_{4.5}$ and *B. subtilis* IS38 (σ^K ; K) at $t_{4.5}$ was hybridized with primers yfhP-ex1 (a), yfhQ-ex1 (b), yfhR-ex3 (c), yfhR-ex2 (d, e) and yfhS-ex1 (f). Sequencing reactions (GATC) were performed using the respective oligonucleotides as primers. The positions of the products are indicated by arrows to the right of the sequences.

The 5' end of the *yfhS* transcript was determined by using primer yfhS-ex1. The corresponding signal was obtained with RNA from the cells at t_3 and $t_{4.5}$ (Fig. 3f). The signal was also obtained at $t_{4.5}$ with RNA from the spoIIIG Δ 1 (σ^G) and IS38 (σ^K) mutants, but not from the IS60 (σ^E) mutant (Fig. 3f). These results show that *yfhS* is transcribed by $E\sigma^E$. The -35 (GCGTATA) and -10 (CAAACAAT) regions spaced 15 bp apart found upstream of P6 (Table 3 and Fig. 4b) are very similar to those of the σ^E consensus [ZHATAXX for the -35 region and CATACAHT for the -10 region and a spacing of 14 bp (Z is T or G, H is A or C, X is A or T); Losick & Stragier, 1992; Haldenwang, 1995].

Insertional inactivation of *yfhP*, *yfhQ*, *yfhS*, *yfhR* and *sspE*, and measurement of β -galactosidase activity

Disruptants YFHPd, YFHQd, YFHSd, YFHRd and SSPEd were constructed as described in Methods. Most of the mutants did not exhibit changes in the tested phenotypes compared to the wild-type strain, the exception being the growth rate of YFHRd. In Schaeffer medium the growth rate of YFHRd was about two times lower than that of the wild-type (see Fig. 6a). Since the growth rate of YFHRd on minimal medium was,

however, nearly identical to that of the wild-type strain (data not shown), the *yfhR* gene product seems not to be essential.

An insertion of a pMUTIN1 derivative results in transcriptional fusion with *lacZ*. We measured the β -galactosidase activities of all mutants in Schaeffer medium. Transcription of *yfhP*, *yfhQ* and *yfhR* was observed during exponential growth (Fig. 5a, b), consistent with the results of Northern blot and primer extension analyses. The maximum activity of YFHRd was approximately 80 units (mg protein)⁻¹, whereas that of YFHQd was about sixfold lower (Fig. 5a, b). These results support the transcription of *yfhR* from four promoters (P2, P3, P4 and P5), confirming the primer extension results (Fig. 4a, b). We also constructed transcriptional *lacZ* fusion strains AMP2 and AMP345 with the P2 and the P3, P4 and P5 promoters, respectively, at the *amyE* locus. These fusion strains expressed higher *lacZ* activity than the control (AMLZ) (Fig. 5e), thus providing evidence of the P2-P5 promoters. Transcription of *yfhS* was observed during sporulation (Fig. 5b), consistent with the results of Northern blot and primer extension analyses (Figs 2d and 3f). Moreover, the spoIIID mutant did not exhibit *yfhS* expression (Fig. 5d). Since spoIIID encodes a

Fig. 4. Nucleotide sequences around the putative promoter regions. Nucleotide numbers 1–221 in panel (a) and 1–536 in panel (b) correspond to regions 25015–25235 and 26123–26658, respectively, of the sequence deposited in the DDBJ database as D85082. The deduced amino acid sequence of YfhS (b), the methionine residues corresponding to the putative translational start codons of YfhP (a), YfhQ (a) and YfhR (b), and the C-terminal region of YfhQ (b) are shown in single-letter code. Potential ribosome-binding sites are boxed and a putative T2 terminator is indicated by arrows. Transcriptional start points and directions are shown by capital letters and hooked arrows, respectively. The –10 and –35 sequences of P1 and P6 are underlined; those of P2, P3 and P5 are overlined. The promoter sequence of P4 was not assigned. Asterisks and converging arrowheads indicate stop codons and the deduced stem-loop structure, respectively.

sporulation transcription factor that modulates the expression of both $E\sigma^E$ and $E\sigma^K$ dependence genes (Halberg & Kroos, 1994), this result also supports the theory that *yfhS* is transcribed by $E\sigma^E$ RNA polymerase. We examined sporulation and germination, spore heat resistance and transcription of *sspE* in YFHSD. No significant difference between the wild-type and *yfhS*-deficient strains was found (data not shown). Recently, it was reported that *cse60*, which encodes an acidic product comprising only 60 aa, was transcribed by $E\sigma^E$ during sporulation, but a deletion mutant of it did not show any distinctive germination rate or the coat protein profile phenotype compared with the wild-type strain (Henriques *et al.*, 1997). YfhS is also an acidic 74 aa polypeptide and is expressed in the mother cell. There-

fore, the function of the small acidic proteins during the sporulation phase remains unknown.

In the case of SSPED, strong transcription from the $E\sigma^G$ promoter (P7) has been observed during sporulation (Hackett & Serlow, 1987; Fajardo-Cavazos *et al.*, 1991). Weak activity was also detected during the exponential growth phase (Fig. 5a). These results support the Northern blot analysis data (Fig. 2e). The β -galactosidase activity of SSPED at t_{-2} and t_{-1} was approximately fivefold lower than that of YFHRd (Fig. 5a). We constructed *B. subtilis* SELZ, containing a *sspE-lacZ* translational fusion at the *sspE* locus, to confirm whether SspE is translated during this period. β -Galactosidase activity of SELZ was not detected during

Fig. 5. Growth and β -galactosidase activity of various transcriptional fusion strains (a, b and d), a *sspE-lacZ* translational fusion strain (c) and promoter-*lacZ* fusion strains at the *amyE* locus (e). All strains were grown in Schaeffer medium at 37 °C. Open and filled symbols indicate the growth rate and β -galactosidase activity, respectively. (a) Δ , $\blacktriangle$, wild-type; $\square$, $\blacksquare$, YFHRd (*yfhR::lacZ*); $\circ$, $\bullet$, SSPEd (*sspE::lacZ*). (b) Δ , $\blacktriangle$, YFHQd (*yfhQ::lacZ*); $\square$, $\blacksquare$, YFHPd (*yfhP::lacZ*); $\circ$, $\bullet$, YFHSd (*yfhS::lacZ*). (c) Δ , $\blacktriangle$, wild-type; $\circ$, $\bullet$, SELZ (*sspE-lacZ* translational fusion). (d) Δ , $\blacktriangle$, HSIID (*spolIID yfhS::lacZ*); $\circ$, $\bullet$, YFHSd (*yfhS::lacZ*). (e) $\blacktriangle$, AMLZ (control); $\bullet$, AMP2 (*P2::lacZ*); $\blacksquare$, AMP345 (*P3-P4-P5::lacZ*).

the exponential growth phase (Fig. 5c). Therefore, *sspE* was co-transcribed with *yfhQ* and/or *yfhR*, but not translated.

The lack of the *yfhP* gene product affects the transcription of both the *yfhQ-yfhR-sspE* and *yfhP* operons

Our data suggest that *yfhP*, *yfhQ* and *yfhR* are expressed during early vegetative growth. To determine whether or not the *yfhP* gene product has some effect on the

expression of *yfhQ* and *yfhR*, we performed Northern blot analysis with RNAs derived from YFHPd. Transcripts of 2.6, 1.5 and 1.3 kb in YFHPd reproducibly increased compared to the wild-type strain at t_{-2} and $t_{-1.5}$ (Fig. 6a). The quantities of the 1.5 and 1.3 kb transcripts in YFHPd at $t_{-1.5}$ were 3.4- and 2.1-fold higher than those in the wild-type strain, respectively (Fig. 7). The relative activity of P2 in YFHPd was approximately 5.3-fold at t_{-2} (Figs 6a and 7). These results suggest that YfhP may influence the transcription of the *yfhQ-yfhR-sspE* operon during the exponential

Fig. 6. Effect of the *yfhP* mutation on the transcription of both the *yfhQ-yfhR-sspE* and *yfhP* operons. (a) Northern blot analysis was performed with the *yfhR*-specific RNA probe. Each lane contained 10 μ g total RNA from *B. subtilis* wild-type strain 168 at t_{-2} (lane 1), t_{15} (2), t_{-1} (3) and $t_{0.5}$ (4), or *B. subtilis* YFHPd (*yfhP*) at t_{-2} (5), t_{15} (6), t_{-1} (7) and $t_{0.5}$ (8). (b) Quantitative primer extension analysis was performed with the *yfhP*-ex1 primer and 50 μ g total RNA samples from *B. subtilis* 168 (lanes 1–3) and *B. subtilis* YFHPd (4–6) at t_{-2} (1 and 4), t_{15} (2 and 5) and t_{-1} (3 and 6).

growth phase. As shown in Figs 6(b) and 7, the transcript initiated at P1 in YFHPd had increased approximately 2.5-fold at t_{-2} compared to the wild-type strain. These data indicate that the transcription of *yfhP* is regulated by its own product.

We searched for recognizable sequences or structures around the promoter regions. A possible stem-loop structure ($\Delta G = -15.4$ kcal mol $^{-1}$), located just upstream of the putative Shine–Dalgarno sequence of *yfhP*, was found (Fig. 4). It is not still clear whether this structure is a regulatory element, such as an operator, with respect to the expression of both the *yfhQ-yfhR-sspE* and *yfhP* operons. A similar case has been reported in the *B. subtilis* major autolysin operon which includes *cwlB* (*lytC*) encoding a 50 kDa N-acetylmuramoyl-L-alanine amidase (Kuroda & Sekiguchi, 1991; Kuroda *et al.*, 1992; Lazarevic *et al.*, 1992). The product of *lytR*, which is transcribed divergently from the *cwlB* operon, acts as a negative regulator of the expression of both the *lppX* (*lytA*)–*cwbA* (*lytB*)–*cwlB* (*lytC*) and *lytR* operons (Lazarevic *et al.*, 1992).

Fig. 7. Effect of the *yfhP* mutation on the transcriptional activity of both the *yfhQ-yfhR-sspE* and *yfhP* operons. Reverse transcripts or hybridization signals were quantified by PhosphorImager or NIH Image analyses, respectively, and the relative ratio of YFHPd (*yfhP*) to the wild-type strain for each signal was calculated. P1 indicates the P1-initiated transcript in Fig. 6(b). P2, P3 and P5 indicate the mRNA species corresponding to the 2.6, 1.5 and 1.3 kb transcripts in Fig. 6(a), respectively. Hatched, open, dotted and filled bars indicate the relative promoter activities at t_{-2} , t_{15} , t_{-1} and $t_{0.5}$, respectively.

In this study, we have shown that the *yfhQ*, *yfhR* and *sspE* genes of *B. subtilis* form an operon for which the *yfhP* gene product plays a regulatory role. In our future studies, we aim to determine the physiological roles of this operon.

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