hag expression in *Bacillus subtilis* is both negatively and positively regulated by ScoC

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In *Bacillus subtilis*, motility and chemotaxis require the expression of *hag*, which encodes flagellin. This gene is transcribed by the σ^D form of RNA polymerase and is regulated by a group of proteins called transition state regulators (TSRs). Our studies show that *hag* transcription is negatively regulated by the transition state regulator ScoC, by binding to its promoter. Furthermore, ScoC, indirectly, also positively regulates *hag* by increasing the availability of σ^D by downregulating the levels of the anti- σ^D -factor FlgM. We further show that the positive regulation by ScoC predominates over the negative regulation.

INTRODUCTION

In *Bacillus subtilis*, the *hag* gene, which codes for flagellin, the structural protein of flagella, is transcribed by the σ^D -dependent RNA polymerase (Mirel & Chamberlin, 1989; Ordal *et al.*, 1993). This gene is transcribed as a monocistronic mRNA and shows a temporal pattern of expression that peaks at the end of the exponential phase of growth followed by a decrease in transcript levels as sporulation proceeds (Mirel *et al.*, 2000). In general, temporal regulation of many genes involved in post-exponential-phase processes such as motility, chemotaxis, extracellular enzyme synthesis and sporulation is coordinated by regulatory proteins called transition state regulators (TSRs) such as AbrB, ScoC and SinR (Smith, 1993; Strauch *et al.*, 1989). Regulation of genes is also brought about by the interplay of sigma factors with their cognate anti-sigma factors (Hecker & Volker, 1998; Helmann, 1999; Hughes & Mathee, 1998). For example, in *B. subtilis*, the motility genes are transcribed at the post-exponential phase of growth by the σ^D form of RNA polymerase. Until then, σ^D is kept sequestered by the anti- σ^D factor, FlgM; it is subsequently released to transcribe the motility genes (Helmann, 1999; Hughes & Mathee, 1998; Mirel *et al.*, 1994). FlgM inhibits the activity of σ^D RNA polymerase by the sequestration of σ^D and also by destabilizing the existing σ^D -holoenzyme (Bertero *et al.*, 1999; Helmann, 1999; Hughes & Mathee, 1998). The transition state regulator ScoC is a negative regulator of protease production and sporulation that binds to a consensus DNA sequence, 5'-RATANTATY-3' (Henner *et al.*, 1988; Kallio *et al.*, 1991; Perego & Hoch, 1988). ScoC has also been reported to be a positive regulator of motility genes

(Caldwell *et al.*, 2001), which are mainly transcribed by the σ^D -dependent RNA polymerase (Ordal *et al.*, 1993). This conclusion has been derived from the transcription profiling of *scoC* mutant cells (*scoC4*), which showed that most of the motility genes, including *hag*, are transcribed at lower levels in the *scoC* mutant as compared to that in the wild-type (Caldwell *et al.*, 2001). We were thus interested in understanding the regulation of *hag* by ScoC.

METHODS

Bacterial strains, plasmids and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* DH5 α was used as an intermediate host for plasmid constructions using standard cloning techniques (Sambrook *et al.*, 1989). Both *E. coli* DH5 α and *B. subtilis* cells were grown at 37 °C in LB medium unless specified otherwise. Transformations in *B. subtilis* were performed using the protoplast transformation protocol (Bron, 1990). Where necessary, antibiotics were added to the following final concentrations: for *B. subtilis*, chloramphenicol, kanamycin and spectinomycin were at 5, 10 and 100 $\mu\text{g ml}^{-1}$, respectively; for *E. coli*, ampicillin was at 100 $\mu\text{g ml}^{-1}$.

PCR. PCRs were carried out using either 100 pg plasmid DNA or 2 ng genomic DNA as template. The PCRs were performed in a reaction volume of 50 μl , with 5 units of *Taq* DNA polymerase and 50 pmol of each primer in buffer supplied by the manufacturer (MBI Fermentas). The PCRs contained a final concentration of 1.5 mM MgCl_2 and 200 μM of each dNTP. In general, the PCR was performed for 30 cycles with an initial denaturation step at 95 °C for 5 min and a final extension step at 72 °C for 10 min. The temperature and time for the denaturation, annealing and extension steps varied depending on the form of DNA, the T_m of the primers used and the length of the target sequence; the conditions used for each primer pair are mentioned in the plasmid construction paragraphs below. Oligonucleotides (Table 2) were synthesized at Microsynth AG, Balgach, Switzerland.

Plasmid constructions

Construction of pMhag'-lacZ. A 549 bp DNA fragment (–188 to +361 from the transcription start of *hag*) containing the promoter

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Abbreviations: EMSA, electrophoretic mobility shift assay; NBT/BCIP, nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate.

Table 1. Plasmids and strains used in this study

Plasmid/strain	Description or genotype*	Source or reference†
Plasmids		
pMUTIN4	Integration vector designed for systematic inactivation of coding sequences and for analysis of unknown gene's promoter expression pattern via the β -galactosidase reporter	BGSC (Vagner <i>et al.</i> , 1998)
pRB381	<i>E. coli</i> - <i>Bacillus</i> replicative, multi-copy vector for translational fusion with the β -galactosidase gene, ' <i>lacZ</i> , Km ^r Ap ^r	BGSC (Bruckner, 1992)
pIC56	<i>E. coli</i> - <i>Bacillus</i> shuttle vector	BGSC (Steinmetz & Richter, 1994)
pMhag'- <i>lacZ</i>	pMUTIN4 bearing a 549 bp insert (-189 to +360) containing <i>hag</i> promoter and first 90 codons of <i>hag</i> ORF fused with <i>lacZ</i>	This study
phag- <i>lacZ</i>	pRB381 bearing a 361 bp insert (-189 to +172) of <i>hag</i> promoter region containing H1 (5'-GATATTAAT-3') and H2 (5'-CACAAATATT-3'), <i>rbs</i> and first 27 codons of <i>hag</i> ORF fused with the <i>lacZ</i>	This study
phag- <i>lacZ</i> -H _P	phag- <i>lacZ</i> with H _P (5'-GATATAGGG-3')	This study
phag- <i>lacZ</i> -H _Q	phag- <i>lacZ</i> with H _Q (5'-CGGCCCCGGG-3')	This study
pIC-scoC	pIC56 with 1.65 kb DNA fragment containing <i>scoC</i>	This study
pflgM- <i>lacZ</i>	pRB381 bearing a 151 bp insert (-106 to +45) of <i>flgM</i> promoter region containing <i>rbs</i> and ATG in translation fusion with <i>lacZ</i>	This study
<i>B. subtilis</i> strains		
168	<i>trpC2</i>	BGSC
168 : hag'- <i>lacZ</i>	168 with <i>hag'</i> - <i>lacZ</i> chromosomal integration	This study
168 : hag'- <i>lacZ</i> /pIC-scoC	168 : hag'- <i>lacZ</i> bearing pIC-scoC	This study
168/phag- <i>lacZ</i>	168 bearing phag- <i>lacZ</i>	This study
168/phag- <i>lacZ</i> -H _P	168 bearing phag- <i>lacZ</i> -H _P	This study
168/phag- <i>lacZ</i> -H _Q	168 bearing phag- <i>lacZ</i> -H _Q	This study
168/pflgM- <i>lacZ</i>	168 bearing pflgM- <i>lacZ</i>	This study
JH642	<i>trpC2 pheA1</i>	BGSC
JH642/phag- <i>lacZ</i>	JH642 bearing phag- <i>lacZ</i>	This study
1A764	JH642 <i>flgMΔ80</i>	BGSC (Mirel <i>et al.</i> , 1994)
1A764/phag- <i>lacZ</i>	1A764 bearing phag- <i>lacZ</i>	This study
168scoC	<i>trpC2 scoC::Sp^r</i>	Lab stock (Kodgire <i>et al.</i> , 2006)
168scoC : hag'- <i>lacZ</i>	168scoC with <i>hag'</i> - <i>lacZ</i> chromosomal integration	This study
168scoC/pflgM- <i>lacZ</i>	168scoC bearing pflgM- <i>lacZ</i>	This study

*Km^r, kanamycin resistance; Ap^r, ampicillin resistance; Sp^r, spectinomycin resistance; Cm^r, chloramphenicol resistance. Mutated nucleotides are shown in bold.

†BGSC, *Bacillus* Genetic Stock Center.

and the first 90 codons of the *hag* ORF (Mirel & Chamberlin, 1989) was PCR amplified from genomic DNA of *B. subtilis* 168 with primers KKR211 and KKR212 (Table 2) (95 °C/1 min, 40 °C/1 min, 72 °C/40 s) and fused in translational frame to the *E. coli lacZ* gene, at the *Hind*III and *Bam*HI sites, in the integrative plasmid pMUTIN4 (Table 1) (Vagner *et al.*, 1998) to give pMhag'-*lacZ* (Table 1).

Construction of H_A in pBluescript SK+. To create the mutated H1 site, H_A (5'-CCGCTGCAG-3'), two PCR products were obtained from pMhag'-*lacZ* with primers KKR211/KKR293 (95 °C/1 min, 42 °C/1 min, 72 °C/30 s) and KKR292/KKR212 (Table 2) (95 °C/1 min, 40 °C/1 min, 72 °C/30 s). The first product was digested with *Hind*III and *Pst*I and cloned into pBluescript SK+. The second PCR product was digested with *Pst*I and *Bam*HI and cloned downstream of the first PCR product to give pBluescript SK+ containing H_A. The mutation in H_A was confirmed by *Pst*I digestion as this site was introduced in the primers to create the mutation. This recombinant plasmid was used as template to amplify H_A with primers KKR113/KKR114 (Table 2) (95 °C/1 min, 40 °C/1 min, 72 °C/20 s) for electrophoretic mobility shift (EMSA) studies.

Construction of H_Q in pBluescript SK+. To create the mutated H2 site, H_Q (5'-CGGCCCCGGG-3'), two PCR products were obtained from pMhag'-*lacZ* with primers KKR211/KKR322 (95 °C/1 min, 42 °C/1 min, 72 °C/30 s) and KKR321/KKR212 (Table 2) (95 °C/1 min, 40 °C/1 min, 72 °C/30 s). The first product was digested with *Hind*III and *Sma*I and cloned into pBluescript SK+. The second PCR product was digested with *Sma*I and *Bam*HI and cloned downstream of the first PCR product to give pBluescript SK+ containing H_Q. The mutation in H_Q was confirmed by digestion with *Sma*I as this site was introduced in the primers to create the mutation. This recombinant plasmid was used as template to amplify H_Q with primers KKR292/KKR320 (Table 2) (95 °C/1 min, 46 °C/1 min, 72 °C/20 s) for EMSA studies.

Construction of phag-*lacZ*. To construct phag-*lacZ*, a DNA fragment of 361 bp (-189 to +172) containing the *hag* promoter and the first 27 codons of the *hag* ORF (Mirel & Chamberlin, 1989) was PCR amplified from pMhag'-*lacZ* (Table 1) with primers KKR211/KKR337 (Table 2) (95 °C/1 min, 42 °C/1 min, 72 °C/30 s). The amplified product was digested with *Hind*III/*Bam*HI and fused in

Table 2. Primers used in this study

Primer	Sequence (5'–3')*	Restriction site†
KKR113	GCGTCGACGGATTTTTTATTTTGT	<i>SalI</i>
KKR114	GCAGGATCCACATTGTTTGTTCCT	<i>BamHI</i>
KKR211	GTGAAGCTTGAAATTGACGCCCCA	<i>HindIII</i>
KKR212	CGCGGATCCAAACGTTGAAGGAT	<i>BamHI</i>
KKR262	CGTGTGACACAGGACTATTCGTAGGC	<i>SalI</i>
KKR292	CCGCTGCAGGATGTAGCCGGGAGG	<i>PstI</i>
KKR293	GATCTGCAGCGGGGATTGTCTCTGATTTTG	<i>PstI</i>
KKR296	CCAAAGCTTCACTTCGCAGCCCGCTG	<i>HindIII</i>
KKR306	GGAGGATCCATCTTTTTTCTCCTTATTAG	<i>BamHI</i>
KKR307	CCAGTCGACGGTACCCCTTCTATGCGC	<i>SalI</i>
KKR311	GCATATTCATATGAAAATCAATCAATTTGG	<i>NdeI</i>
KKR312	GCATCTCGAGTTGCTTTTTATAAAATTAATC	<i>XhoI</i>
KKR320	CGTGGATCCGTTGTTTGAAGACAAACG	<i>BamHI</i>
KKR321	CGACCCGGGCGAGCGCTTAACACACTG	<i>SmaI</i>
KKR322	CGTCCCGGGCCGGTTAATTCTCATTGTTTT	<i>SmaI</i>
KKR327	GCTCCCGGGGATGTAGCCGGGAGG	<i>SmaI</i>
KKR328	TATATCGGATTGTCTCTGATTTTGTTAAT	
KKR337	GCAGGATCCATGTTCTTTTGGCTCGCACT	<i>BamHI</i>

*Nucleotides in bold are complementary to the genome sequence.

†Underlined in the corresponding sequence.

translational frame to the *E. coli lacZ* gene in the replicative multi-copy plasmid pRB381 (Bruckner, 1992) to give phag-lacZ (Table 1). Plasmid pRB381 is derived from plasmid pUB110 (Bruckner, 1992) and the copy number of pUB110 in *B. subtilis* is about 50 per chromosome (Bron, 1990).

Construction of phag-lacZ-H_P. To create H_P (5'-GATATAGGG-3'), in which the 3'-end of H1 was mutated, two PCR products were obtained from pMhag'-lacZ with primers KKR327/KKR212 (95 °C/1 min, 40 °C/1 min, 72 °C/30 s) and KKR211/KKR328 (Table 2) (95 °C/1 min, 42 °C/1 min, 72 °C/30 s). The first product was digested with *SmaI* and *BamHI* and cloned into pBluescript SK+. The second PCR product was digested with *HindIII* and cloned upstream of the first PCR product to give H_P in pBluescript SK+. The latter was used as the template to reamplify the *hag* promoter containing the H_P site with primers KKR211/KKR337 (Table 2) and cloned in translational fusion with *lacZ* in pRB381 to give phag-lacZ-H_P (Table 1). pBluescript SK+ carrying H_P was also used to amplify H_P with primers KKR113/KKR114 (Table 2) for EMSA with ScoC.

Construction of phag-lacZ-H_Q. The *hag* promoter containing the H_Q site was amplified from the recombinant pBluescript SK+ plasmid carrying H_Q (described above) with primers KKR211/KKR337 and cloned in translational fusion with *lacZ* in pRB381 to give phag-lacZ-H_Q (Table 1).

Construction of pflgM-lacZ. A 151 bp DNA fragment (–106 to +45 from the transcription start of *yvyF*) containing the P_{D-1} promoter and the ATG of *yvyF* (Mirel *et al.*, 1994) was PCR amplified from genomic DNA of *B. subtilis* 168 with primers KKR296/KKR306 (Table 2) (95 °C/1 min, 48 °C/1 min, 72 °C/20 s), digested with *HindIII* and *BamHI* and fused in translational frame with the *E. coli lacZ* gene in pRB381 to give plasmid pflgM-lacZ (Table 1).

Construction of pIC-scoC. A 1.65 kb DNA fragment containing the promoter, ORF and transcription terminator of *scoC* (Perego & Hoch, 1988) was amplified from genomic DNA of *B. subtilis* 168 with

primers KKR262/KKR307 (Table 2) (95 °C/1 min, 48 °C/1 min, 72 °C/1 min 40 s) and cloned at the *SalI* site in plasmid pIC56 (copy no. ~10) (Steinmetz & Richter, 1994) to give pIC-scoC (Table 1).

Assay for β -galactosidase in *B. subtilis*. The strains were grown at 37 °C in Penassay broth to stationary phase (OD₆₀₀ ~2.0) and the β -galactosidase activities were determined (Nicholson & Setlow, 1990). All the β -galactosidase assays presented in this work were performed in triplicate. The activities presented are the mean of triplicate values. At least three independent experiments were performed and a representative result is shown. We ensured that the *lacZ* results were not due to differences in copy numbers between the various strains. We compared the copy number of pRB381-derived plasmids by dot-blots, which were very similar in all the strains (data not shown).

Electrophoretic mobility shift assay. EMSA was performed as described in the Roche gel shift manual (<http://www.roche-applied-science.com/pack-insert/3353591a.pdf>). End-labelling of the probe was carried out with digoxigenin-11-ddUTP (DIG-ddUTP) using terminal transferase in 20 μ l reaction buffer containing 0.2 M potassium cacodylate, 0.25 M Tris/HCl, pH 6.6, 0.25 mg BSA ml⁻¹ and 5 mM CoCl₂ at 37 °C for 15 min, and then 2 μ l 0.2 M EDTA (pH 8.0) was added to terminate the reaction. Binding reactions were carried out with 10 nM DIG-labelled probe and ScoC (1 μ M) in 20 μ l reaction buffer containing 20 mM HEPES, pH 7.6, 1 mM EDTA, 10 mM (NH₄)₂SO₄, 1 mM DTT, Tween 20 (0.2 %, w/v), 30 mM KCl, 1 μ g poly(dI-dC), and 0.1 μ g poly-L-lysine at 37 °C for 15 min. For the competition reaction, protein was first incubated in presence of 100-fold molar excess of competitor DNA (unlabelled specific DNA) followed by incubation with the labelled probe. The bound product was electrophoresed on a 5 % polyacrylamide gel in 0.25 $\times$ Tris/borate/EDTA buffer at 4 °C. The gel was electroblotted onto a nylon membrane and treated with anti-DIG-alkaline phosphatase conjugate. DNA was detected with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) in AP buffer (100 mM Tris/HCl, pH 9.5, 100 mM NaCl, 5 mM MgCl₂) until visible bands were observed.

Cloning, expression and purification of FlgM, and raising of anti-FlgM antibodies. The gene coding for FlgM (Mirel *et al.*, 1994) was PCR amplified from genomic DNA of *B. subtilis* 168 with primers KKR311/KKR312 (Table 2) (95 °C/1 min, 45 °C/1 min, 72 °C/30 s) and cloned into plasmid pET28a (Novagen) at the *Nde*I and *Xho*I sites to give pFlgM. The cloning strategy was designed to provide a His-tag at the C terminus of the protein. pFlgM was transformed into *E. coli* BL21(DE3) and FlgM was induced with 0.1 mM IPTG. *E. coli* BL21(DE3)/pFlgM was inoculated in 5 ml LB broth and grown overnight at 37 °C in an orbital shaker at 200 r.p.m. Then 50 µl of the overnight grown culture was used to inoculate 5 ml fresh LB broth. The culture was grown at 37 °C in an orbital shaker at 200 r.p.m. for 2.5 h, until the OD₆₀₀ reached 0.5–0.6. Freshly prepared IPTG was added to a final concentration of 0.1 mM and incubation continued for 40 min. Rifampicin was added to a final concentration of 150 µg ml⁻¹ and incubation continued for 2.5 h. Cells were harvested by centrifugation and the pellet was washed with 1 ml buffer containing 25 mM Tris/HCl, pH 7.4. The pellet was resuspended in 500 µl buffer containing 25 mM Tris/HCl, pH 7.4, and cells were lysed by lysozyme treatment at 30 °C for 15 min, followed by sonication at 4 °C. The cell lysate was centrifuged to separate supernatant and pellet and samples were analysed by SDS-PAGE (data not shown).

FlgM was affinity purified from the supernatant on a Ni-NTA resin as described in the Qiagen Ni-NTA spin kit manual. Input sample was prepared in 50 mM phosphate buffer, pH 8.0, 0.3 M NaCl and 10 mM imidazole and loaded on a spin column. The column was washed with the above buffer containing 100 mM imidazole (six column volumes). The protein sample was eluted with 150 µl buffer containing 1 M imidazole. Samples were analysed by SDS-PAGE to determine purity and recovery of the protein. The purified protein, which showed a single band with purity greater than 95% (data not shown), was used for raising polyclonal anti-FlgM antibodies in rabbits (by Bangalore Genei, Bangalore, India).

We tested the ability of the anti-FlgM antibodies to detect FlgM in extracts of *B. subtilis* 168. The cells were grown at 37 °C for 6 h and the presence of FlgM was tested by Western blot analysis (Sambrook *et al.*, 1989) with the anti-FlgM antibodies. A single band of ~10 kDa was clearly detected in wild-type cells that was missing in the *flgM* mutant 1A764 (Mirel *et al.*, 1994), in which 80 of the 88 amino acids have been deleted (see Fig. 4A).

Western blot analysis of FlgM. Equal number of cells, corresponding to OD₆₀₀ ~3.0, of *B. subtilis* 168 and 168scoC strains from an overnight culture were seeded into 25 ml Penassay broth and grown with shaking at 37 °C. The growth rates of both strains were identical. Samples (5 ml) corresponding to equal numbers of cells were harvested at 5 h (*t*₁) and 6 h (*t*₂) relative to *t*₀ (4 h of growth; time of entry into stationary phase) and centrifuged. The pellet was heated at 95 °C for 15 min in 30 µl SDS-loading buffer, loaded onto a 15% SDS-polyacrylamide gel and electrophoresed onto a nitrocellulose membrane. The membrane was probed with rabbit anti-FlgM antibodies (raised against purified FlgM; see above); bands were detected using an alkaline phosphatase-linked secondary antibody and NBT/BCIP (Sambrook *et al.*, 1989).

RESULTS

hag is negatively regulated by ScoC

To study the involvement of ScoC in the transcription of *hag*, a 549 bp DNA fragment (–188 to +361 from the transcription start site of *hag*) containing the *hag* promoter and the first 90 codons of the *hag* ORF (Mirel &

Chamberlin, 1989) was fused to the *E. coli lacZ* gene in an integrative plasmid, pMUTIN4 (Vagner *et al.*, 1998), to give pMhag'-lacZ (Table 1) and transformed into *B. subtilis* 168 and 168scoC, a *scoC* disruptant (Kodgire *et al.*, 2006), to yield 168:hag'-lacZ and 168scoC:hag'-lacZ (Table 1). The strains were grown with shaking at 37 °C in Penassay broth to stationary phase OD₆₀₀ ~2.0) and the β-galactosidase activities were determined (Nicholson & Setlow, 1990). We observed an almost twofold increase in the *hag* promoter activity in 168scoC:hag'-lacZ (435 ± 15 Miller units) as compared to that in wild-type (225 ± 10 Miller units) (Table 3A), indicating that ScoC negatively regulates *hag* transcription. This result was in contrast to that observed from the transcription profiles of *B. subtilis* 168 and *scoC4*, which demonstrated decreased *hag* transcription in *scoC4* (Caldwell *et al.*, 2001).

To determine if the negative regulation of *hag* by ScoC is due to a direct binding of ScoC to the *hag* promoter region (Mirel & Chamberlin, 1989), we searched for putative ScoC binding sites using BioEdit (<http://www.mbio.ncsu.edu/BioEdit/bioedit.html>). This search revealed the presence of two sites located between –11 and –2 (H1, 5'-GATATTAAT-3') and between +103 and +112 (H2, 5'-CACAAATATT-3') (Fig. 1) with an 8/9 and 7/9 match with the consensus ScoC binding site (5'-RATANTATY-3'), respectively (Caldwell *et al.*, 2001). The H1 site overlaps with the –10 region of the bipartite *hag* promoter and the H2 site is located in the *hag* ORF (Fig. 1). We therefore tested the ability of ScoC to bind to these sites by EMSA.

ScoC binds to the *hag* promoter region

Binding reactions were carried out with 1 µM ScoC and a 150 bp DNA probe (10 nM) containing either the H1 or H2 site. The two probes were obtained by PCR amplification from pMhag'-lacZ with primers KKR113/KKR114 and

Table 3. *hag* reporter expression in *B. subtilis* strains

Strains	β-Galactosidase activity (Miller units)*
A	
168:hag'-lacZ	225 ± 10
168scoC:hag'-lacZ	435 ± 15
168:hag'-lacZ/pIC-scoC	410 ± 20
B	
168/phag-lacZ	2000 ± 50
168/phag-lacZ-H _P	16500 ± 150
168/phag-lacZ-H _Q	17000 ± 150
C	
JH642/phag-lacZ	1700 ± 25
1A764/phag-lacZ	3700 ± 50

*The β-galactosidase assays were performed as described in Methods. The activities shown are the mean of triplicate values. At least three independent experiments were performed and a representative result is shown.

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gcagcgttatccagcgcgatgtgatctccgcattatcctca
                                     -35
caaaaaaagtgcaggatTTTTTTTATTTTgtattaacaaa
      -10  H1      +1
atcagagacaatccgatattaatgatgtagccgggagga
ggcgcaaaagactcagccagttacaaaataagggcaciaa
                                     rbs
ggacgtgccttaacaacatattcagggagggaacaaaaca
                                     H2
1  - atg aga att aac cac aat att gca gcg ctt
                                     31
31 - aac aca ctg aac cgt ttg tct tca aac aac

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Fig. 1. Sequence of the *hag* promoter region (Mirel & Chamberlin, 1989) and location of ScoC binding sites. ScoC binding sites H1 and H2 are indicated by the boxed nucleotides. Nucleotides in bold in these sites are identical to the ScoC consensus sequence, 5'-RATANTATY-3' (Caldwell *et al.*, 2001). +1 denotes the transcription start site. The -10 sequence (5'-ccgatat-3'), the -35 sequence and ribosome-binding site (*rbs*) are underlined.

KKR292/KKR320, respectively (Table 2). Fig. 2(A, B) shows that both the DNA probes, containing the H1 and H2 sites respectively, were retarded in the presence of ScoC (lane 2) as compared to in its absence (lane 1). The retardation was significantly reduced with a 100-fold molar excess of unlabelled probe (lane 3), showing the specificity of binding. Thus, we conclude that ScoC is capable of binding to both the sites in the *hag* promoter region.

To further confirm the specificity of binding of ScoC to H1 and H2, we mutated H1 and H2 to H_A (5'-CCGCTGCAG-

3') and H_Q (5'-CGGCCCGGG-3'), respectively, and then performed the EMSA with ScoC. The *hag* promoter containing the mutated H1 (H_A) or mutated H2 (H_Q) sites was amplified from pBluescript SK+ carrying either H_A or H_Q with primers KKR113/KKR114 and KKR292/KKR320, respectively, and used as probes for the binding experiments. Fig. 2(A, B), lane 4 shows that mutation of either of the ScoC binding sites significantly reduced the binding of ScoC to the *hag* promoter region. Furthermore, the mutated probes could not compete for binding of ScoC to the respective wild-type probes (Fig. 2A, B, lane 5), indicating that H1 and H2 are bona fide binding sites for ScoC. Thus, our results are strongly in support of a direct transcriptional regulation of *hag* by ScoC.

Mutation of the H1 or H2 site in the *hag* promoter region relieves repression

To investigate whether mutation of either the H1 or H2 site led to relief from repression, we made three constructs, phag-lacZ, phag-lacZ-H_P and phag-lacZ-H_Q (Table 1), carrying the wild-type *hag* promoter and mutated H1 and H2 sites respectively. To construct phag-lacZ, a DNA fragment of 361 bp (-189 to +172) containing the *hag* promoter and the first 27 codons of the *hag* ORF (Mirel & Chamberlin, 1989) was cloned in translational frame to the *E. coli lacZ* gene in the replicative multi-copy plasmid pRB381 (Table 1) (Bruckner, 1992). The constructs phag-lacZ-H_P and phag-lacZ-H_Q were identical to phag-lacZ in all respects except for the mutation in the H1 (H_P) or H2 (H_Q) site, respectively. Unlike H_A, in which all the bases of H1 were mutated and which was used for the binding experiments with ScoC, the H_P site in phag-lacZ-H_P contained mutations in only four bases from the 3'-end of H1 (5'-GATATAGGG-3'). This was done so as to keep the

Fig. 2. (A) EMSA of ScoC with DIG-labelled *hag* probe containing H1 or H_A or H_P sites. See Methods for details of the assay conditions. Lanes: 1, labelled H1; 2, labelled H1 + ScoC; 3, labelled H1 + ScoC + 100× unlabelled H1; 4, labelled H_A + ScoC; 5, labelled H1 + ScoC + 100× unlabelled H_A; 6, labelled H_P + ScoC; 7, labelled H1 + ScoC + 100× unlabelled H_P. (B) EMSA of ScoC with DIG-labelled *hag* probe containing H2 and H_Q sites. Lanes: 1, labelled H2; 2, labelled H2 + ScoC; 3, labelled H2 + ScoC + 100× unlabelled H2; 4, labelled H_Q + ScoC; 5, labelled H2 + ScoC + 100× unlabelled H_Q.

–10 sequence of the promoter intact (Fig. 1). Binding studies confirmed that, similar to H_A, ScoC is unable to bind H_P (Fig. 2A, lane 6) and the partially mutated probe could not compete for binding of ScoC to the wild-type probe (Fig. 2A, lane 7).

The two plasmids, phag-lacZ-H_P and phag-lacZ-H_Q, were introduced into *B. subtilis* 168 to give 168/phag-lacZ-H_P and 168/phag-lacZ-H_Q (Table 1) and the promoter activities were compared with that in 168/phag-lacZ (phag-lacZ in *B. subtilis* 168, Table 1). The promoter activities in 168/phag-lacZ-H_P and 168/phag-lacZ-H_Q were $16\,500 \pm 150$ and $17\,000 \pm 150$ Miller units, respectively, as compared to 2000 ± 50 Miller units in 168/phag-lacZ (Table 3B), clearly indicating that the mutation of either the H1 or the H2 site led to loss of repression.

The level of relief from repression upon mutation of either the H1 or the H2 site was almost eightfold higher as compared to a twofold relief in 168scoC:hag'-lacZ. Similar twofold relief was observed when the multicopy plasmid phag-lacZ was introduced into 168scoC and *hag* reporter expression was compared with that in 168/phag-lacZ (data not shown). This suggested to us that *hag* is subject to both negative and positive regulation and that in 168scoC:hag'-lacZ the extent of relief in repression is blunted by the loss of positive regulation.

Based on the transcription profiling of *scoC4*, in which motility genes were shown to be downregulated, it was proposed that ScoC positively regulates the availability of σ^D by downregulating FlgM activity (Caldwell *et al.*, 2001). FlgM has previously been shown to be a negative regulator of *hag* expression (Fredrick & Helmann, 1996). We confirmed that FlgM indeed negatively regulates *hag* transcription by measuring *hag* expression in an *flgM* mutant background (1A764, Table 1) and compared its activity with that in the isogenic wild-type strain, JH642 (Table 1). We measured the effect of FlgM on *hag* expression in the JH642 background instead of in the 168 background, avoiding intricacies of moving the in-frame *flgM* deletion into 168 without any selectable marker, since strain 1A764, an *flgM* mutant, was created by deleting 80 of the 88 amino acids in the FlgM ORF (Mirel *et al.*, 1994). The plasmid phag-lacZ was introduced into *B. subtilis* JH642 and *B. subtilis* 1A764 to give JH642/phag-lacZ and 1A764/phag-lacZ (Table 1), respectively, and the promoter activities were determined. The β -galactosidase activity in 1A764/phag-lacZ was 3700 ± 50 Miller units, more than twofold higher as compared to JH642/phag-lacZ (1700 ± 25 Miller units; Table 3C). A similar increase in *hag* promoter activity in the *flgM* mutant background was previously reported by Fredrick & Helmann (1996).

flgM is transcriptionally regulated by ScoC

To show experimentally that ScoC downregulates FlgM, we assessed *flgM* transcription in wild-type *B. subtilis* 168 and 168scoC. *flgM* is the second gene of an operon that

contains four ORFs, *yvyF* (*orf* 139), *flgM*, *yvyG* (*orf* 160) and *flgK*, and is transcribed from a single promoter, P_{D-1} (Mirel *et al.*, 1994). The P_{D-1} promoter and the ATG of *yvyF* (hereafter referred to as the *flgM* promoter) was cloned in translational frame with the *E. coli lacZ* gene in pRB381 (Bruckner, 1992) to give plasmid pflgM-lacZ (Table 1). pflgM-lacZ was transformed into *B. subtilis* 168 and 168scoC to yield 168/pflgM-lacZ and 168scoC/pflgM-lacZ (Table 1) and the β -galactosidase activities were determined (Nicholson & Setlow, 1990) between 4 h (*t*₀) and 6 h (*t*₂) of growth (*t*₀ corresponds to entry into stationary phase), as *flgM* expression has been observed to be maximal during this stage of growth (Liu & Zuber, 1998). The growth rates of both strains were identical. The promoter activity in 168scoC/pflgM-lacZ was at least twice that in 168/pflgM-lacZ (Fig. 3), indicating that ScoC negatively regulates *flgM* expression. The increase in expression of *flgM* in 168scoC is also reflected in the levels of FlgM protein as determined by Western blot analysis using polyclonal anti-FlgM antibodies raised against purified FlgM. Fig. 4(B) shows that the level of FlgM is at least two to three times higher in 168scoC as compared to that in wild-type cells when measured at 5 h (*t*₁) and 6 h (*t*₂). We conclude that the negative regulation of *flgM* is also reflected in the levels of the FlgM protein. These results thus provide experimental support that ScoC positively regulates *hag* expression by downregulating the levels of FlgM, leading to enhanced σ^D activity. The fourfold difference observed between the relief from repression when either of the sites is mutated and the relief in repression in 168scoC:hag'-lacZ also suggests that positive regulation of *hag* by ScoC predominates.

If this were the case we reasoned that if ScoC was introduced on a multi-copy plasmid in wild-type cells, then the *hag* promoter activity should be higher than in cells carrying a single copy. For this purpose, *B. subtilis* 168 was transformed with a multi-copy plasmid, pIC-scoC

Fig. 3. *flgM* reporter expression in *B. subtilis* 168/pflgM-lacZ (□) and 168scoC/pflgM-lacZ (■). *t*₀ is the time of entry into stationary phase. Data shown (means $\pm$ SE from triplicate values) are from a representative experiment of at least three independent experiments.

Fig. 4. (A) Confirmation of anti-FlgM specificity of anti-FlgM polyclonal antibodies by Western blot analysis of *B. subtilis* 168 and 1A764 (*flgM* mutant). (B) Detection of FlgM in *B. subtilis* 168 and 168scoC.

(Table 1), encoding ScoC, to give 168:hag'-lacZ/pIC-scoC, and the promoter activity of *hag* was compared with that in 168:hag'-lacZ. The *hag* promoter activity of 168:hag'-lacZ/pIC-scoC was 410 ± 20 Miller units and was twice that of 168:hag'-lacZ (225 ± 10 Miller units) (Table 3A). This clearly supports the contention that the positive effect of ScoC is predominant.

DISCUSSION

Transcription profiling has shown that 29 out of 55 motility and chemotaxis genes are affected by the transition state regulator ScoC (Caldwell *et al.*, 2001). This is a testament to the crucial role played by ScoC in the regulation of motility genes in *B. subtilis*. To date, the exact mechanism for this regulation is not known, although it was thought that ScoC regulates these genes by indirectly regulating the availability of σ^D via a pathway that involves SinR (Caldwell *et al.*, 2001). SinR is expressed from a dicistronic operon, *sinIR*. The product of the first gene, SinI, post-translationally antagonizes the activity of SinR by converting SinR from its active tetrameric state to an inactive SinI-SinR heterodimer. ScoC represses *sinI* transcription (Kallio *et al.*, 1991; Shafikhani *et al.*, 2002), resulting in elevated levels of active SinR and thus leading to increased levels of σ^D (Caldwell *et al.*, 2001), probably via decreasing FlgM activity. This model is supported by the observation that deletion of *flgM* could restore motility in a *sinR* null mutant (Fredrick & Helmann, 1996). However, we have demonstrated that ScoC downregulates FlgM and thereby positively controls the availability of σ^D and therefore motility gene expression.

Our observation that ScoC is a repressor of *hag* expression is contrary to the report of Caldwell *et al.* (2001), wherein, from transcriptional profiling experiments, they reported decreased *hag* expression in a *scoC* mutant background. In general microarray results are quite reliable; however, accurate measurement of absolute expression levels is often not possible for various reasons such as suboptimal design or choice of probes, inconsistent sequence fidelity of the spotted microarrays, variability of differential expression and discrepancy in fold-change calculation (Kothapalli *et al.*, 2002; Draghici *et al.*, 2006). Our results clearly suggest that ScoC specifically binds to two sites in the *hag* promoter region and that repression of *hag* is relieved in the absence of ScoC. The presence of two ScoC binding sites in the *hag* promoter region suggests that, as in the case of the *nprE*, *aprE* and *sinI* promoters (Kallio *et al.*, 1991), ScoC may manifest its function through the formation of a bent repression loop as proposed by several investigators (Smith, 1993; Strauch & Hoch, 1992, 1993), thereby affecting promoter clearance. In addition ScoC may regulate transcription initiation of *hag* by preventing open complex formation, as the H1 site overlaps with the -10 region of the promoter (Fig. 1). CodY, another regulator of *hag*, monitors the general nutritional state of the cell by sensing intracellular GTP levels (Fisher, 1999; Ratnayake-Lecamwasam *et al.*, 2001). It is known to repress *hag* expression in rich media, and repression of *hag* is relieved approximately twofold in a *codY* mutant background. Interestingly, the H2 site of *hag* overlaps with the CodY binding site (Bergara *et al.*, 2003). Therefore, it is possible that mutation of H2 might affect the binding of CodY to the *hag* promoter region and additionally contribute to the relief in repression of *hag*.

Fig. 5. Model for *hag* regulation by ScoC in *B. subtilis*.

The difference in the extent of relief from repression in 168scoC as compared to that in 168/phag-lacZ- H_P and 168/phag-lacZ- H_Q suggests that *hag* transcription is both negatively and positively regulated by ScoC. The positive regulation is manifested by increased availability of σ^D as a result of decreased levels of FlgM. A model for *hag* regulation is presented in Fig. 5. We envisage that such a dual control by ScoC is most likely aimed at ‘fine-tuning’ *hag* expression levels to suit the cell’s needs in different environmental conditions, such as efficient nutrient acquisition, avoidance of toxic substances, translocation to optimal colonization sites and dispersal in the environment.

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REFERENCES

- Bergara, F., Ibarra, C., Iwamasa, J., Patarroyo, J. C., Aguilera, R. & Marquez-Magana, L. M. (2003). CodY is a nutritional repressor of flagellar gene expression in *Bacillus subtilis*. *J Bacteriol* **185**, 3118–3126.
- Bertero, M. G., Gonzales, B., Tarricone, C., Cecilian, F. & Galizzi, A. (1999). Overproduction and characterization of the *Bacillus subtilis* anti-sigma factor FlgM. *J Biol Chem* **274**, 12103–12107.
- Bron, S. (1990). Plasmids. In *Molecular Biological Methods for Bacillus*, pp. 75–139. Edited by C. R. Harwood & S. M. Cutting. New York: Wiley.
- Bruckner, R. (1992). A series of shuttle vectors for *Bacillus subtilis* and *Escherichia coli*. *Gene* **122**, 187–192.
- Caldwell, R., Sapolsky, R., Weyler, W., Maile, R. R., Causey, S. C. & Ferrari, E. (2001). Correlation between *Bacillus subtilis* scoC phenotype and gene expression determined using microarrays for transcriptome analysis. *J Bacteriol* **183**, 7329–7340.
- Draghici, S., Khatri, P., Eklund, A. C. & Szallasi, Z. (2006). Reliability and reproducibility issues in DNA microarray measurements. *Trends Genet* **22**, 101–109.
- Fisher, S. H. (1999). Regulation of nitrogen metabolism in *Bacillus subtilis*: vive la difference! *Mol Microbiol* **32**, 223–232.
- Fredrick, K. & Helmann, J. D. (1996). FlgM is a primary regulator of σ^D activity, and its absence restores motility to a *sinR* mutant. *J Bacteriol* **178**, 7010–7013.
- Hecker, M. & Volker, U. (1998). Non-specific, general and multiple stress resistance of growth-restricted *Bacillus subtilis* cells by the expression of the σ^B regulon. *Mol Microbiol* **29**, 1129–1136.
- Helmann, J. D. (1999). Anti-sigma factors. *Curr Opin Microbiol* **2**, 135–141.
- Henner, D. J., Ferrari, E., Perego, M. & Hoch, J. A. (1988). Location of the targets of the *hpr-97*, *sacU32(Hy)*, and *sacQ36(Hy)* mutations in upstream regions of the subtilisin promoter. *J Bacteriol* **170**, 296–300.
- Hughes, K. T. & Mathee, K. (1998). The anti-sigma factors. *Annu Rev Microbiol* **52**, 231–286.
- Kallio, P. T., Fagelson, J. E., Hoch, J. A. & Strauch, M. A. (1991). The transition state regulator Hpr of *Bacillus subtilis* is a DNA-binding protein. *J Biol Chem* **266**, 13411–13417.
- Kodgire, P., Dixit, M. & Rao, K. K. (2006). ScoC and SinR negatively regulate *epi* by corepression in *Bacillus subtilis*. *J Bacteriol* **188**, 6425–6428.
- Kothapalli, R., Yoder, S. J., Mane, S. & Loughran, T. P., Jr (2002). Microarray results: how accurate are they? *BMC Bioinformatics* **3**, 22.
- Liu, J. & Zuber, P. (1998). A molecular switch controlling competence and motility: competence regulatory factors ComS, MecA, and ComK control σ^D -dependent gene expression in *Bacillus subtilis*. *J Bacteriol* **180**, 4243–4251.
- Mirel, D. B. & Chamberlin, M. J. (1989). The *Bacillus subtilis* flagellin gene (*hag*) is transcribed by the σ^{28} form of RNA polymerase. *J Bacteriol* **171**, 3095–3101.
- Mirel, D. B., Lauer, P. & Chamberlin, M. J. (1994). Identification of flagellar synthesis regulatory and structural genes in a σ^D -dependent operon of *Bacillus subtilis*. *J Bacteriol* **176**, 4492–4500.
- Mirel, D. B., Estacio, W. F., Mathieu, M., Olmsted, E., Ramirez, J. & Marquez-Magana, L. M. (2000). Environmental regulation of *Bacillus subtilis* σ^D -dependent gene expression. *J Bacteriol* **182**, 3055–3062.
- Nicholson, W. L. & Setlow, P. (1990). Sporulation, germination, and outgrowth. In *Molecular Biological Methods for Bacillus*, pp. 442–444. Edited by C. R. Harwood & S. M. Cutting. New York: Wiley.
- Ordal, G. W., Márquez-Magaña, L. M. & Chamberlin, M. J. (1993). Motility and Chemotaxis. In *Bacillus subtilis and Other Gram-positive Bacteria: Biochemistry, Physiology, and Molecular Genetics*, pp. 765–784. Edited by A. L. Sonenshein, J. A. Hoch & R. Losick. Washington, DC: American Society for Microbiology.
- Perego, M. & Hoch, J. A. (1988). Sequence analysis and regulation of the *hpr* locus, a regulatory gene for protease production and sporulation in *Bacillus subtilis*. *J Bacteriol* **170**, 2560–2567.
- Ratnayake-Lecamwasam, M., Serró, P., Wong, K. W. & Sonenshein, A. L. (2001). *Bacillus subtilis* CodY represses early-stationary-phase genes by sensing GTP levels. *Genes Dev* **15**, 1093–1103.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Shafikhani, S. H., Mandic-Mulec, I., Strauch, M. A., Smith, I. & Leighton, T. (2002). Postexponential regulation of *sin* operon expression in *Bacillus subtilis*. *J Bacteriol* **184**, 564–571.
- Smith, I. (1993). Regulatory proteins that control late-growth development. In *Bacillus subtilis and Other Gram-positive Bacteria: Biochemistry, Physiology, and Molecular Genetics*, pp. 785–800. Edited by A. L. Sonenshein, J. A. Hoch & R. Losick. Washington, DC: American Society for Microbiology.
- Steinmetz, M. & Richter, R. (1994). Plasmids designed to alter the antibiotic resistance expressed by insertion mutations in *Bacillus subtilis*, through *in vivo* recombination. *Gene* **142**, 79–83.
- Strauch, M. A. & Hoch, J. A. (1992). Control of postexponential gene expression by transition state regulators. In *Biology of Bacilli: Application to Industry*, pp. 105–121. Edited by R. H. Doi & M. McGloughlin. Stoneham, MA: Butterworth-Heinemann.
- Strauch, M. A. & Hoch, J. A. (1993). Transition-state regulators: sentinels of *Bacillus subtilis* post-exponential gene expression. *Mol Microbiol* **7**, 337–342.
- Strauch, M. A., Spiegelman, G. B., Perego, M., Johnson, W. C., Burbulys, D. & Hoch, J. A. (1989). The transition state transcription regulator *abrB* of *Bacillus subtilis* is a DNA binding protein. *EMBO J* **8**, 1615–1621.
- Vagner, V., Dervyn, E. & Ehrlich, S. D. (1998). A vector for systematic gene inactivation in *Bacillus subtilis*. *Microbiology* **144**, 3097–3104.

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