Spo0A positively regulates *epr* expression by negating the repressive effect of co-repressors, SinR and ScoC, in *Bacillus subtilis*

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Bacillus subtilis under nutritional deprivation exhibits several physiological responses such as synthesis of degradative enzymes, motility, competence, sporulation, etc. At the onset of post-exponential phase the global response regulator, Spo0A, directly or indirectly activates the expression of genes involved in the above processes. These genes are repressed during the exponential phase by a group of proteins called transition state regulators, e.g. AbrB, ScoC and SinR. One such post-exponentially expressed gene is *epr*, which encodes a minor extracellular serine protease and is involved in the swarming motility of *B. subtilis*. Deletion studies of the upstream region of *epr* promoter revealed that *epr* is co-repressed by transition state regulators, SinR and ScoC. Our study shows that Spo0A positively regulates *epr* expression by nullifying the repressive effect of co-repressors, SinR and ScoC. We demonstrate via *in vitro* mobility shift assays that Spo0A binds to the upstream region of *epr* promoter and in turn occludes the binding site of one of the co-repressor, SinR. This explains the mechanism behind the positive regulatory effect of Spo0A on *epr* expression.

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1. Introduction

In *Bacillus subtilis* the response regulator, Spo0A, is a key regulatory element in the post-exponential phase phenomenon like initiation of sporulation, exoprotease production, autolysin production, induction of motility and development of competence (Strauch *et al*. 1990; Errington 1993). *epr*, a minor extracellular serine protease, is necessary for the swarming motility of the organism (Sloma *et al*. 1988; Murudkar *et al*. 2006). *epr* is expressed during the post-exponential phase in response to stationary-phase signals (Dixit *et al*. 2002). The expression as well as the timing of expression of *epr* is regulated during the exponential phase by two transition state regulators (TSRs), SinR and ScoC. These two TSRs negatively regulate the *epr* expression by the process of co-repression (Kodgire *et al*. 2006). During post-exponential phase, most of the protease expressing genes are activated directly or indirectly by Spo0A (Strauch *et al*. 1990; Olmos *et al*. 1996; Sanchez and Olmos 2004; Ogura *et al*. 2004; Kodama *et al*. 2007). Spo0A, in its phosphorylated form, functions as an activator or repressor of a variety of genes by binding to a 7 bp consensus sequence (5′-TGNCGAA-3′) called '0A' box (Strauch *et al*. 1990). In this study we demonstrate that similar to other exoproteases, *epr* expression is positively regulated by Spo0A. Unlike other exoproteases, the positive regulatory role of Spo0A on *epr* expression is by negating the repressive effect of co-repressors, SinR and ScoC. Our results further indicate that Spo0A negates repression by binding to the upstream

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region of *epr* promoter and in turn occluding the binding site of SinR.

2. Methods

2.1 *Bacterial strains, plasmids and growth conditions*

Bacterial strains and plasmids used in this study are listed in table 1. *E. coli* DH5α strain was used as an intermediate host for plasmid constructions using standard cloning techniques (Sambrook *et al*. 1989). Both *E. coli* DH5α and *B. subtilis* 168 cells were grown at 37°C in Luria Bertani (LB) medium unless specified otherwise. Protoplast method of transformation was performed to introduce plasmid DNA into *B. subtilis* strains (Bron 1990). Wherever necessary, antibiotics were added to the following final concentrations: for *B. subtilis*, kanamycin was at 10 $\mu g mL^{-1}$, whereas for *E. coli*, ampicillin was added to a final concentration of 100 $\mu g mL^{-1}$. Sequence integrity of all plasmid constructions were verified by sequencing at Macrogen Inc (Seoul 153–781, Korea) using appropriate PCR primers (table 2). The presence of plasmid in the transformed *B. subtilis* strains was confirmed by plasmid isolation (Bron 1990) followed by PCR with appropriate primers.

2.2 *Polymerase chain reaction*

Polymerase chain reactions (PCR) were carried out using 2 ng of genomic DNA as template. The PCR reactions were performed in a volume of 50 µL with 5 units of *Taq* DNA polymerase and 50 pmol of each primer in a buffer supplied by the manufacturer (MBI Fermentas, Lithuania). The PCR reactions contained a final concentration of 1.5 mM $MgCl_2$ and 200 µM of each dNTP. In general, the PCR reaction was

Table 1. List of plasmids and strains

Plasmids and Strains	Description or genotype	Source or reference†
Plasmids		
pRB381	*E. coli - Bacillus* replicative, multicopy vector for translational fusion with the β-galactosidase gene, *'lacZ*, Km^r, Ap^r	BGSC (Bruckner 1992)
pUZ	pRB381 bearing a 500 bp insert (-464 to +33) containing the putative 0A box, SinR and ScoC binding sites, *epr* promoter, RBS and ATG in translation fusion with the *lacZ* gene	This study
pHZ	pRB381 bearing a 341 bp insert (−308 to +33) containing the *epr* promoter, RBS and ATG in translation fusion with the *lacZ* gene	Laboratory Stock (Gupta and Rao 2009)
pS200Z	pRB381 bearing a 655 bp insert containing the *epr* promoter (−422 to +33), RBS, ATG and an additional 200 bp insertion between SinR and ScoC binding site in translation fusion with the *lacZ* gene	Laboratory Stock (Kodgire *et al*. 2006)
pBRP200	pBR322 bearing the *epr* promoter region (−760 to +33) with an additional 200 bp DNA insertion between the SinR and ScoC binding sites	Laboratory Stock (Kodgire *et al*. 2006)
pU200Z	pUZ with a 200 bp DNA insertion between the SinR and ScoC binding sites	This study
pSpo0ABD	pET28a bearing *spo0ABD* (380 bp) that codes for C-terminal DNA binding domain of Spo0A	This study
pScoC	pET28a bearing *scoC* (600 bp) that codes for ScoC	Laboratory Stock (Kodgire *et al*. 2006)
pSinR	pET43.1b bearing *sinR* (342 bp) that codes for SinR	Laboratory Stock (Kodgire *et al*. 2006)
Strains		
B. subtilis 168	*trpC2*	BGSC
1S10	*trpC2, spo0A12*	BGSC
168/UZ	*B. subtilis* 168 bearing pUZ	This study
1S10/UZ	*B. subtilis* 1S10 bearing pUZ	This study
168/HZ	*B. subtilis* 168 bearing pHZ	Laboratory Stock (Gupta and Rao 2009)
1S10/HZ	*B. subtilis* 1S10 bearing pHZ	This study
168/U200Z	*B. subtilis* 168 bearing pU200Z	This study
1S10/U200Z	*B. subtilis* 1S10 bearing pU200Z	This study

Ap^r, ampicillin resistance; Km^r, kanamycin resistance.

† BGSC, *Bacillus* Genetic Stock Centre.

Table 2. List of primers

Primers	Sequence (5′ - 3′) †	Restriction site(s) ‡
KKR35	**GTTGGGTAACGCCAGG**	
KKR36	CTTAGGATCC**ATGATTCATCTCC**	*BamH*I
KKR73	CGAGATCTCTGCAGA**TCGACACCATAGC**	*Bgl*II and *Pst*I
KKR74	CGAGATCTCTGCAGA**AAGCGGCAATCA**	*Bgl*II and *Pst*I
KKR127	**CGCTTTGCGTGACGGATTATC**	
KKR259	GCACTCGAG**AGAAGCCTTATGCTC**	*Xho*I
KKR279	**TACTGACTATAAGAACTAATTCTT**	
KKR280	**AATAACTACACGTCCTAATTCATC**	
KKR291	GCATCCATGG**CTAGCAGCCAGCCTGAA**	*Nco*I

† Nucleotides in bold are complimentary to the genome sequence.

‡ Restriction enzymes sites are underlined in the corresponding sequence.

performed for 30 cycles with initial denaturation step at 95°C for 5 min and the final extension step at 72°C for 10 min. The time and temperature for 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 time and temperature for denaturation, annealing and extension for each primer pair are mentioned under specific plasmid constructions discussed below. Oligonucleotides (table 2) were synthesized at Microsynth AG, Balgach, Switzerland.

2.3 *Construction of pUZ and pU200Z*

For constructing pUZ, a 525 bp DNA fragment, region between −464 to +33 from the transcription start site of *epr* (Sloma *et al*. 1988; Bruckner *et al*. 1990; Dixit *et al*. 2002) (figure 1) was PCR-amplified from *B. subtilis* 168 genomic DNA using primers KKR74/KKR36 (table 2). To construct pU200Z, a 700 bp DNA fragment, region from −464 to +33 from the transcription start site of *epr* with an additional 200 bp DNA insertion between SinR and ScoC binding sites (figure 1), was PCR-amplified from plasmid pBRP200 (table 1) using primers KKR74/KKR36 (table 2). The time and temperature for denaturation, annealing and extension for the above constructs were 1 min/95°C, 1 min/61°C and 40 s/72°C respectively. The PCR products were fused independently in translational frame to the *E. coli lacZ* gene at the *Pst*I and *BamH*I sites in the vector pRB381 (table 1) (Bruckner 1992) to give pUZ and pU200Z respectively (table 1). The constructs were verified by PCR with primers KKR74/KKR35 (table 2) and by sequencing at Macrogen Inc., Seoul 153-781, Korea.

2.4 *Cloning, expression and purification of Spo0ABD*

A 380 bp DNA fragment containing *spo0ABD* was PCR-amplified from *B. subtilis* 168 genomic DNA using primers KKR259/KKR291 (table 2). The amplified product was cloned at *Nco*I and *Xho*I sites in the vector pET28a (Novagen) to give pSpo0ABD (table 1). pSpo0ABD was transformed into *E. coli* BL21 (DE3) and protein was induced with 0.1 mM IPTG and affinity purified on a Ni-NTA resin as described in the Qiagen Ni-NTA spin kit manual. The purified protein showed a single band with purity greater than 95% (data not shown).

2.5 *Electrophoretic mobility shift assay*

Electrophoretic mobility shift assay (EMSA) was performed as described in the Roche gel shift manual (Roche Diagnostics GmbH, Mannheim, Germany, *www.roche-applied-science.com/pack-insert/3353591a.pdf*). End-labelling of the probe was carried out with digoxigenin-11-dUTP (DIG-dUTP) using terminal transferase in a 20 µL reaction buffer containing 0.2 M potassium cacodylate, 0.25 M Tris-Cl, pH 6.6, 0.25 mg/mL BSA and 5 mM $CoCl_2$ at 37°C for 15 min, and then 2 µL of 0.2 M EDTA (pH 8.0) was added to terminate the reaction. Binding reactions were carried out with a 10 nM DIG-labelled probe and protein of required concentration in a 20 µL reaction buffer containing 20 mM HEPES, pH 7.6, 1 mM EDTA, 10 mM $(NH_4)_2SO_4$, 1 mM dithiothreitol, Tween 20 (0.2% [w/v]), 30 mM KCl, 1 µg poly d(I-C), and 0.1 µg poly-L-lysine at 37°C for 15 min. For the competition reaction, protein was first incubated in the presence of a 100-fold molar excess of competitor DNA (unlabelled specific DNA) followed by incubation with the labelled probe. The bound product was electrophoresed on a pre-ran 5% polyacrylamide gel in 0.25X Tris-borate-EDTA buffer at 80 Volts in 4°C for 3–4 h. Migration was monitored by Bromophenol blue and Xylene cyanol mobility on the gel. Gel was electroblotted onto a Nylon membrane and DNA was detected with nitroblue tetrazolium/5-bromo-4-chloro-3-indolylphosphate (NBT/BCIP) in ALP buffer (100 mM Tris-Cl, pH 9.5, 100 mM

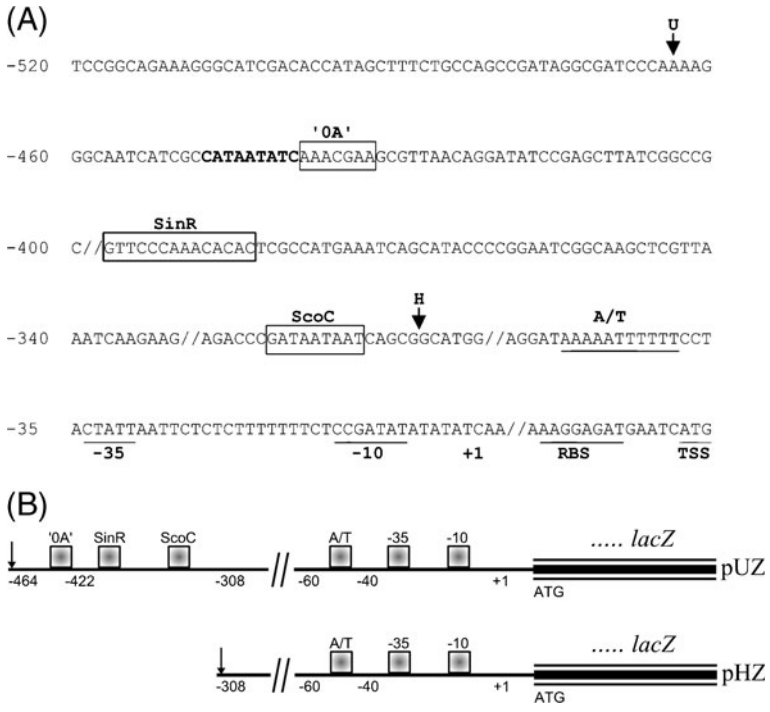

Figure 1. (**A**) Upstream sequence of *epr* promoter region (*http://genolist.pasteur.fr/SubtiList/*). Positions of the deletions have been marked with arrows. Positions of putative '0A' box, SinR and ScoC binding sites have been boxed. The position of −35 and −10 elements, RBS, A/T rich region and translation start site (TSS) have been underlined. The transcription start site has been denoted as +1. Positions are marked with respect to the transcription start site. Nucleotides marked in bold indicate the putative binding site of ScoC. (**B**) Schematic representation of deletion of upstream region of *epr* promoter. Arrows pointing downwards represents the site of deletion.

NaCl, 5 mM $MgCl_2$) until visible bands were observed. The blot was transferred to a fresh Petri plate containing 20 mL of 1X PBS + 80 µL of 0.5 M EDTA to terminate the reaction.

2.6 *β-Galactosidase assay*

The strains were grown at 37°C in Penassay broth to an OD A_{600}~2.0 and the β-galactosidase activities were determined (Nicholson and Setlow 1990). All the β-galactosidase assays presented in this work were performed in triplicates. Activities presented are the mean value of triplicates. Copy numbers of pRB381-derived plasmids were compared by dot blot hybridization to ensure that *lacZ* results are not due to the difference in copy numbers between various strains (data not shown).

3. Results

3.1 *Spo0A positively regulates epr expression*

To determine if the *epr* expression is dependent on Spo0A, we constructed a plasmid carrying the *epr* promoter region that includes the sequence from −464 to +33 from the transcription start site, fused to the *E. coli lacZ* gene (pUZ; figure 1 and table 1). pUZ was introduced into wild-type *B. subtilis* 168 and in *B. subtilis* 1S10 (*spo0A* mutant) to yield 168/UZ and IS10/UZ respectively (table 1). The region −464 to +33 of *epr* promoter was chosen because no change in the promoter activity was observed upon further deletion upstream to the *epr* promoter (data not shown). The promoter activity of 1S10/UZ was analysed and compared with the wild-type cells 168/UZ (table 1). We observed a complete loss of *epr* expression in 1S10/UZ as compared to its expression in 168/UZ (figure 2). This clearly indicates that Spo0A positively regulates *epr* expression. Careful examination of −464 to +33 region of *epr* promoter revealed a 7 bp sequence (figure 1; region −464 to −440), which closely resembles the consensus Spo0A binding sequence ('0A' box) and could be a probable site for Spo0A binding.

3.2 *Spo0A negates the repressive effect of co-repressors, SinR and ScoC*

TSRs, SinR and ScoC, co-repress the *epr* expression by binding to their respective sites that lie between −422 and −308 from the transcription start site (figure 1) (Kodgire *et al*. 2006). Deletion of −464 to −308 bp from *epr* promoter region (pHZ; figure 1 and table 1) results in an increase in

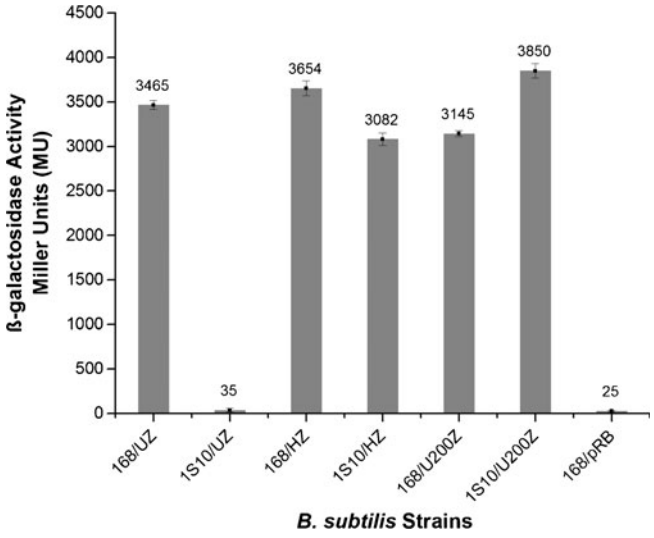

Figure 2. *epr* promoter activity in *B. subtilis* 168 and in *spo0A* mutant (1S10) background. All the β- galactosidase assays were conducted at the onset of OD A_{600}~2.0 (Methods) at which maximal *epr* promoter activity is observed. Assays were performed in triplicate. Activities presented are the mean value of triplicates. Error bars represents the SD of at least three independent experiments.

epr expression comparable to wild-type, 168/UZ (figure 2) (Kodgire *et al*. 2006, Gupta and Rao 2009). Interestingly, the region from −464 to −308 harbours a putative '0A' box as well as TSRs (SinR and ScoC) binding sites. To decipher the mechanism of positive regulatory effect of Spo0A on *epr* expression, we introduced the construct pHZ (lacking Spo0A and TSR's binding sites) into a *spo0A* mutant to give 1S10/HZ (table 1). While we observed complete loss of *epr* expression in the absence of Spo0A in 1S10/UZ strain, there was almost no loss in *epr* promoter activity in 1S10/HZ cells as compared to the wild-type cells, 168/HZ (figure 2). Hence, this suggests that in the absence of repression (due to the removal of SinR and ScoC binding sites); *epr* expression is independent of positive regulatory effect of Spo0A. It further indicated that unlike other exoproteases, in the case of *epr*, Spo0A does not function as a classical activator; otherwise, in the absence of Spo0A, *epr* promoter activity would have been much lower in 1S10/HZ as compared to 168/HZ.

The proximity of SinR and ScoC binding sites is critical for them to function as co-repressors of *epr* expression. Increasing the distance between the two binding sites by an unrelated DNA of 200 bp (pS200Z; table 1) led to a complete loss of repression (Kodgire *et al*. 2006). We presumed that if a 200 bp sequence was similarly inserted between the SinR and ScoC binding sites in plasmid pUZ, then due to the loss of repression, *epr* expression would be Spo0A independent. To test this hypothesis we constructed plasmid pU200Z carrying −464 to +33 region of *epr* promoter with an additional 200 bp DNA sequence between SinR and ScoC binding site (table 1). pU200Z was introduced into wild-type and in a *spo0A* mutant to give 168/U200Z and IS10/U200Z respectively (table 1), and β-galactosidase activity was determined. Figure 2 shows that the *epr* promoter activity was virtually abolished in 1S10/UZ as compared to 168/UZ. In contrast, the *epr* promoter activity in 1S10/U200Z was very similar to the promoter activities in 168/U200Z as well as 168/UZ. This further reinforces the contention that in the absence of repression, Spo0A is not required for *epr* expression. Thus, the above two evidences indicate that Spo0A acts as an anti-repressor of *epr* expression by negating the negative regulatory effect of TSRs, SinR and ScoC.

3.3 *Spo0A binds to the upstream region of epr promoter*

Electrophoresis mobility shift assay (EMSA) was performed to demonstrate the binding of Spo0A onto the upstream region of *epr* promoter. Our initial attempts to show the binding were unsuccessful. This could be due to very low level of phosphorylated form of Spo0A (Spo0A~P) when expressed in *E. coli*. Phosphorylation of Spo0A is required to activate Spo0A (Lewis *et al*. 2002). Interestingly, it has been reported that the removal of 426 bp N-terminal domain of Spo0A (NTD) eliminates the need for phosphorylation of Spo0A for activation. The truncated form of Spo0A (Spo0ABD) is capable of binding to the '0A' boxes (*comK*, *abrB*, *sdpA* and *med*) with efficiencies equivalent to that of the phosphorylated Spo0A (Ireton *et al*. 1993; Rowe-Magnus and Spiegelman 1998; Molle *et al*. 2003). Therefore, we performed binding studies with Spo0ABD. For this purpose, we cloned Spo0ABD in pET28a to give pSpo0ABD (table 1). Spo0ABD (hereafter Spo0A) expressed in *E. coli* was affinity purified on a Ni-NTA column (data not shown). The biological activity of purified Spo0A was verified by performing binding studies with the *abrB* promoter that has experimentally been shown to bind Spo0A (Strauch *et al*. 1990). For this purpose, a 250 bp *abrB* promoter region (−150 to +100 from the transcription start site) was PCR-amplified with primers KKR279/KKR280 (table 2) and end-labelled with digoxigenin (DIG)-11-dUTP. The results in figure 3A show that the migration of the *abrB* probe was significantly retarded in the presence of 1 μM Spo0A (figure 3A, lane 2) as compared to the migration in its absence (figure 3A, lane 1). Retardation was reduced in the presence of a 100-fold molar excess of unlabelled probe (figure 3A, lane 3), indicating the specificity of binding of Spo0A to the *abrB* probe. Having demonstrated the biological activity of Spo0A, we carried out binding studies with 1 μM Spo0A and a 230 bp *epr* promoter region −494 to −264 from the transcription start site

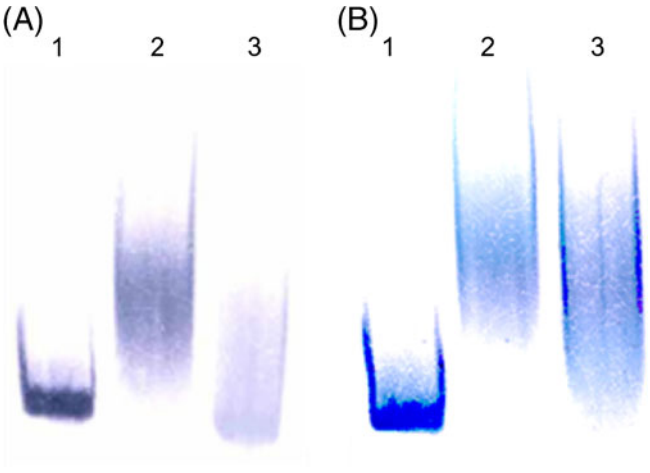

Figure 3. Electrophoresis mobility shift assay (EMSA) of Spo0A onto the upstream promoter regions of *abrB* and *epr* respectively. 1 μM of Spo0A is the optimal concentration standardized to demonstrate Spo0A binding onto the promoters in all the EMSA's. Smearing of the bound DNA fragment is observed in our experiment, this is possibly due to dissociation of protein- DNA complexes during electrophoresis: (**A**) EMSA of Spo0A onto the 250 bp *abrB* promoter region: DIG-labeled *abrB* probe (lane 1); *abrB* probe + 1 μM Spo0A (lane 2) and *abrB* probe + 1 μM Spo0A + 100X unlabeled *abrB* probe (lane 3). (**B**) EMSA of Spo0A onto the 230 bp *epr* promoter: DIG labeled *epr* probe (lane 1); *epr* probe + 1 μM Spo0A (lane 2) and *epr* probe + 1 μM Spo0A + 100X unlabeled *epr* probe (lane 3).

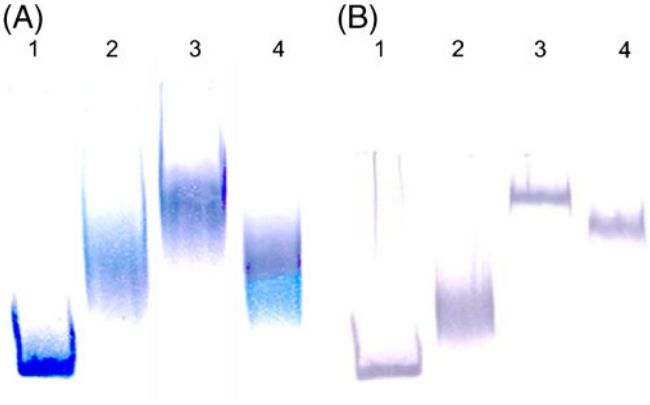

Figure 4. Electrophoresis Mobility Shift Assay (EMSA) of upstream region of *epr* promoter with Spo0A, SinR and ScoC: (**A**) DIG-labelled *epr* probe (lane 1); *epr* probe + 1 μM Spo0A (lane 2); *epr* probe + 10 μM SinR (lane 3) and *epr* probe with 1 μM Spo0A + 10 μM SinR (lane 4). (**B**) DIG-labelled *epr* probe (lane 1); *epr* probe + 1 μM Spo0A (lane 2); *epr* probe + 1 μM ScoC (lane 3) and *epr* probe with 1 μM Spo0A + 1 μM ScoC (lane 4).

(carrying putative '0A' box) (figure 1), which was PCR-amplified using primers KKR73/KKR127 (table 2). The *epr* probe was retarded in the presence of Spo0A (figure 3B, Lane 2) as compared to the probe in its absence (figure 3B, lane 1). Retardation was reduced in the presence of a 100-fold molar excess of unlabelled probe (figure 3B, lane 3) showing the specificity of binding. Thus, the above result shows that Spo0A binds to the *epr* promoter region.

3.4 *Binding of Spo0A occludes the binding site of SinR but not of ScoC*

Competitive binding of Spo0A and co-repressors were performed to examine if upstream bound Spo0A negates the repression by occluding binding site(s) of one or both co-repressors. The rationale behind the experiment was that if Spo0A binds to the upstream region of the *epr* promoter and occludes the binding site of either of the two repressors, then the retardation of the probe with Spo0A in the presence of either SinR or ScoC would be identical to the extent of retardation of the probe observed with Spo0A alone. However, if Spo0A does not occlude the binding sites of either SinR or ScoC, then both Spo0A and SinR or ScoC would bind and a super shift of the probe would be observed. We first tested competitive binding between Spo0A and SinR. The binding studies were carried out on a 230 bp *epr* probe (−494 to −264 from the transcription start site containing Spo0A, SinR and ScoC binding sites; figure 1) with 1 μM Spo0A and 10 μM SinR (obtained from the expression vector pSinR; table 1). The extent of retardation was compared with the retardations of the probe with either Spo0A or SinR alone. To perform binding studies with Spo0A and SinR, the probe was pre-incubated with Spo0A for 15 min followed by the addition of SinR. The results in figure 4A show that the probes treated with Spo0A and SinR independently are retarded to different extents with SinR showing a more pronounced retardation (figure 4A, lanes 2 and 3, respectively). However, in the presence of both Spo0A and SinR, the retardation observed is very similar to the retardation observed with Spo0A alone (figure 4A, lane 4), suggesting that the binding of Spo0A prevented the binding of SinR. To analyse whether the above competition reaction is vice versa, we pre-incubated the probe with SinR for 15 min followed by the addition of Spo0A. If SinR too occludes the binding of Spo0A we expected the retardation in the presence of both the proteins to be similar to the retardation observed in the case of SinR alone. In fact, we observed a faint band at the margin of the well (data not shown). The most probable explanation of this result would be that pre-incubation of SinR did not restrict the binding of Spo0A resulting in a heavy DNA–protein complex, hence restricting the latter to enter the gel.

Similar binding studies with Spo0A and 1 μM ScoC (obtained from the expression vector pScoC; table 1) showed that Spo0A and ScoC bind independently to the *epr*

promoter region (figure 4B, lanes 2 and 3) with the retardation of probe by ScoC being significantly more pronounced than with Spo0A alone (figure 4B, lane 3). However, when the probe is pre-incubated with Spo0A followed by the binding of ScoC, the retardation observed is greater than that of Spo0A alone (figure 4B, lane 4). This, *a priori*, suggests that Spo0A does not prevent binding of ScoC, because if it did, then we would expect that in the presence of both proteins the retardation should be identical to that of Spo0A alone. Unexpectedly, no super shift was also seen; in fact, the retardation of the probe in the presence of both the proteins was slightly less than the retardation observed with ScoC alone. A possible explanation for this anomalous result could be that there exists an additional ScoC binding site in the vicinity of the Spo0A binding site, where the binding of ScoC to this additional site is occluded by Spo0A, but not to the known site located between −319 and −310 (figure 1). Therefore, in the absence of Spo0A, ScoC binds to both of these sites resulting in retardation of the probe as observed in figure 4B, lane 3. However, in the presence of Spo0A and ScoC, only one site is occupied by ScoC resulting in retardation of the probe that is intermediate between the retardation observed for Spo0A or ScoC alone (figure 4B, lanes 2, 3 and 4). Indeed, there does appear to be an additional putative ScoC binding site located between −448 and −439 (figure 1A) that has an 8/9 match with the consensus ScoC binding site. Studies are underway to establish if this additional ScoC binding site is biologically functional.

4. Discussion

The post-exponentially expressed gene, *epr*, encodes one of the minor exoproteases (Sloma *et al*. 1988) and has been shown to be involved in the swarming motility of *B. subtilis* (Murudkar *et al*. 2006). During the exponential phase, expression of *epr* is negatively regulated by the process of co-repression by transition state regulators, SinR and ScoC (Kodgire *et al*. 2006). This study demonstrates that *epr* expression is positively regulated by the post-exponential response regulator, Spo0A. We identified a 40 bp sequence between the −464 and −422 upstream region of the *epr* promoter with a putative binding site for Spo0A (figure 1). Removal of this 40 bp sequence (region −464 to −422; figure 1), resulted in reduction of *epr* expression (Kodgire *et al*. 2006). Interestingly, removal of the 40 bp sequence, SinR and ScoC binding sites (pHZ, figure 1) leads to restoration of *epr* promoter activity (figure 2), suggesting that the 40 bp sequence negates the repressive effect of ScoC and SinR. Furthermore, introduction of construct pHZ into a *spo0A* mutant strain, 1S10, resulted in *epr* promoter activity comparable to wild-type strain 168 (figure 2), thereby making *epr* expression independent of positive regulatory effect of Spo0A. This suggests that the 40 bp sequence is Spo0A-dependent and Spo0A acts as a positive regulator of *epr* expression by acting as an anti-repressor rather than classical activator.

To determine the mechanism of positive regulatory role of Spo0A on *epr* expression we performed the binding of Spo0A onto the *epr* promoter. EMSA conducted on the 230 bp *epr* promoter region (−494 to −264 from the transcription start site of the *epr* promoter; figure 1) shows that Spo0A binds to the upstream region of *epr* promoter (figure 3B). While we were able to show the binding of Spo0A onto the 230 bp *epr* promoter, we were unable to show a direct binding between Spo0A and 40 bp oligo carrying putative '0A' box, i.e. region between −464 to −422 from the transcription start site of the *epr* promoter (data not shown). At present we do not have any viable explanation for this observation.

We were interested in determining whether the upstream bound Spo0A affects the binding of one or both the co-repressors. *In vitro* competitive binding experiments were performed to demonstrate that Spo0A occludes the binding site of SinR but not of ScoC (figure 4). Since SinR and ScoC co-repress the *epr* expression (Kodgire *et al*. 2006), in the absence of one of the negative regulator, SinR, *epr* expression is relieved from repression. Interestingly, the intermediate retardation of the *epr* promoter probe observed in the presence of Spo0A and ScoC (figure 4B, lane 4) suggested the presence of another ScoC binding site in the vicinity of the 40 bp sequence (figure 1A). The above EMSA results will be validated in future by conducting experiments using the specific antibodies to detect the exact nature of the proteins in the shifted bands.

Similar anti-repression property of Spo0A has been reported in Spo0A-dependent stage II sporulation gene, *spoIIG*. The binding sites of Spo0A and SinR overlap at the *spoIIG* promoter and the presence of Spo0A inhibits the binding of SinR and hence anti-represses the transcription of the *spoIIG* gene (Cervin *et al*. 1998). Anti-repression in bacteria can occur by various mechanisms. In one, the anti-repressing protein sequesters the repressor, preventing its binding to DNA. This mechanism has been documented in number of cases; for instance, SinI interacts with SinR and hence prevents the latter from binding to the *aprE* promoter of *B. subtilis* (Bai *et al*. 1993) and MogR regulation in *Listeria* by GmaR (Shen *et al*. 2006). In a second mechanism, the anti-repressor protein does not affect the interaction of the repressor with the promoter, but brings about some conformational changes leading to the bending of DNA, counteracting the effects of repressors without preventing their binding. This mechanism has been observed in *B. subtilis*, where the positive regulatory effect of ComK on its own promoter is attributed to the neutralization of the effect of the negative regulator *rok* without affecting its binding (Smits *et al*. 2007). A third mechanism of anti-repression in bacteria involves occlusion of the repressor binding site by other DNA binding proteins. For example, in *B. subtilis* the binding of CcpA to the *ilvB* promoter prevents binding of the nutritional repressor CodY (Shivers

and Sonenshein 2005). The ability of Spo0A to occlude the binding of SinR on the *epr* promoter, discussed in this article, is an additional example of the third mechanism of anti-repression in bacteria.

In conclusion, the results presented in this study provide new insight into the complex temporal regulatory network of post-exponentially expressed gene, *epr*, in *B. subtilis*. During the post-exponential phase of growth, the phosphorylated form of Spo0A (Spo0A~P) binds to the upstream promoter region of *epr*. Upstream bound Spo0A occludes the binding site of one of the co-repressor, SinR. In the absence of SinR, repression of *epr* expression is not achieved and the transcription of *epr* commences. On the other hand, during the exponential phase, Spo0A is mainly in its non-phosphorylated state (Hoch 1993) and the effective concentrations of the TSRs, SinR and ScoC, are high in the cell (Strauch *et al*. 1989; Smith 1993), thus resulting in the repressed state of *epr* expression.

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