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ScoC and SinR Negatively Regulate *epr* by Corepression in *Bacillus subtilis*

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Negative regulation of *epr* in *Bacillus subtilis* 168 is mediated jointly by both ScoC and SinR, which bind to their respective target sites 62 bp apart. Increasing the distance between the two sites abolishes repression, indicating that the two proteins interact, thereby suggesting a mechanism of corepression.

The expression of many of the genes coding for enzymes such as proteases, amylases, etc. (28), is repressed in the exponential phase by a group of regulatory proteins called "transition state regulators" (15, 27, 34). AbrB, ScoC, and SinR are among the best-characterized transition state regulators (33, 34, 36). Both ScoC and SinR are known to be negative regulators of protease production that bind to a DNA sequence whose consensus sequences appear to be 5′-RATANTATY-3′ (14, 16, 27, 35) and 5′-GNCNCGAAATACA-3′, respectively (12, 31). The active state of SinR is in the tetrameric form, and its activity is antagonized by SinI (3, 6, 21).

We had previously shown that Epr, a minor extracellular protease in *Bacillus subtilis* (5, 32), is transcribed by a σ^D-dependent RNA polymerase and that it is involved in swarm activity (8, 25). We were thus interested in determining how this gene is regulated. In this study, we show that negative regulation of *epr* requires both ScoC and SinR and that their mode of action appears to be through a mechanism of corepression.

Inspection of the sequence upstream of the *epr* promoter revealed the presence of putative ScoC and SinR binding sites, 5′-GATAATAAT-3′ and 5′-GTTCCCAAACACA-3′, respectively (Fig. 1), that display an 8/9 match and a 10/13 match with the consensus binding sites for ScoC and SinR. To determine whether these two sites conferred negative regulation on *epr*, two DNA fragments of 457 bp (−424 to +33) and 343 bp (−310 to +33), with and without the two sites, respectively, and containing the *epr* promoter, ribosome binding site (RBS), and ATG were PCR amplified from pPZ (Table 1) with primers KKR28/KKR36 and KKR103/KKR36 (Table 2). The amplified products were digested with HindIII/BamHI and PstI/BamHI, respectively, fused in the translational frame to the *lacZ* gene in pRB381, a replicative multicopy plasmid (4), to give pSZ and pHZ (Table 1) and then were transformed into *B. subtilis* 168 to give 168-SZ and 168-HZ, respectively. Both strains were grown at 37°C in Penassay broth to the stationary phase (optical density at 600 nm [OD_{600}] of ~2.0), and the β-galactosidase activities were determined (26). The activity in 168-HZ was 3,500 Miller units, as compared to 200 Miller units in 168-SZ, indicating that the region between −422 and −308, containing the putative binding sites for ScoC and SinR, negatively regulates *epr* expression. Further deletions from −308 to −70 did not show any significant change in activity compared to 168-HZ (data not shown).

We then assessed whether ScoC and SinR bind to their respective sites by the electrophoretic mobility shift assay (EMSA). The genes coding for ScoC (27) and SinR (11) were cloned into pET28a and pET43.1b, respectively, expressed in *Escherichia coli* BL21(DE3), and the two proteins were purified on Ni-nitrilotriacetic acid columns. Binding reactions were carried out in a 20-μl reaction mixture at 37°C for 15 min, with either 1 μM ScoC or 12 μM SinR, and a 150-bp DNA containing both binding sites was obtained by PCR amplification from pPZ with primers KKR28 and KKR127 (Table 2) and end labeled with digoxigenin (DIG). (Procedures for DNA labeling, DNA binding, and detection were performed as per Roche Applied Science, catalog no. 3353591.) The reaction mixture was electrophoresed on a 5% polyacrylamide gel and electroblotted onto Nylon membrane, and DNA was detected with nitroblue tetrazolium–5-bromo-4-chloro-3-indolylphosphate (NBT/BCIP). Figure 2A shows that the DNA probe is significantly retarded in the presence of ScoC (lane 2) compared to in its absence (lane 1). The amount of DNA probe showing retardation is significantly reduced, with a 100× molar excess of unlabeled probe (lane 3) showing the specificity of binding. Similarly in Fig. 2B, the probe is retarded in the presence of SinR (lane 2) compared to in its absence (lane 1). The retardation is abolished, with a 100× molar excess of unlabeled probe (lane 3) showing the specificity of binding. Thirty-base-pair oligonucleotides containing either the putative ScoC or SinR binding site also were able to compete with ScoC or SinR binding to the labeled probe, respectively, but not reciprocally (data not shown). We thus conclude that both ScoC and SinR bind to a specific site within the *epr* promoter.

To determine whether ScoC and SinR were involved in the repression of *epr*, we transformed pSZ into 168ΔH and 168ΔR (Table 1), *scoC* and *sinR* disruptants, respectively, to give 168ΔH-SZ and 168ΔR-SZ. The β-galactosidase activities in 168ΔH-SZ, 168ΔR-SZ, and 168-SZ were 3,400, 2,000, and 200 Miller units, respectively, showing that negative regulation of *epr* was dependent on both ScoC and SinR and that neither,

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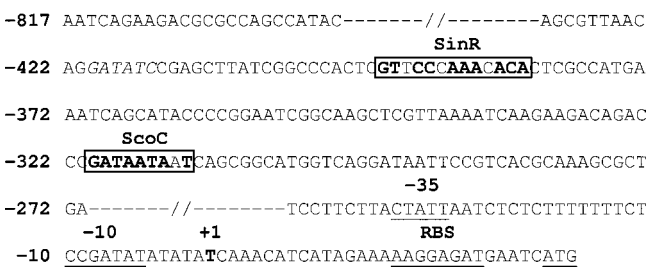

FIG. 1. Sequence of *epr* promoter region (−817 to +33) (5, 8, 30). ScoC and SinR binding sites are indicated by the boxed nucleotides. Nucleotides marked in bold within these sites are identical to the consensus recognition sequence (16, 29).

individually, can fully repress *epr*. This observation thus suggests a mechanism of corepression that may involve interaction of the two proteins. Since SinI is also known to interact with SinR (3), it may regulate *epr* expression by preventing corepression. The reduced level of derepression in 168ΔR-SZ compared to 168ΔH-SZ may be due to a reduction in σ^D levels in the *sinR* disruptant since SinR appears to positively regulate σ^D levels via FlgM (9).

In further support of the idea that both ScoC and SinR were required for repression, we made two constructs, pS_HZ and pS_RZ (Table 1), carrying either a mutated ScoC (5′-GATCC CGGG-3′) or mutated SinR (5′-TTAATATTTATAA-3′) binding site (mutations underlined), respectively, but identical in all other respects to pSZ and assessed whether they independently lead to relief from repression. To create the mutated ScoC binding site, two PCR products were obtained from pPZ with primers KKR67/KKR216 and KKR215/KKR36 (Table 2). The first product was restricted with HindIII and SmaI and cloned into pBluescript SK+. The second PCR product was restricted with SmaI and BamHI and cloned downstream of the first PCR product in pBluescript SK+. The *epr* promoter containing the mutated ScoC binding site was reamplified with primers KKR28/KKR36 (Table 2) and cloned in pRB381 to give pS_HZ. pS_RZ was similarly constructed from two PCR products obtained from pPZ with primers KKR67/KKR218 and KKR217/KKR36 (Table 2) and sequentially cloned into pBR322 at HindIII/SspI and SspI/BamHI, respectively. The fused product was reamplified with primers KKR28/KKR36 and cloned in pRB381 to give pS_RZ. Mutations of ScoC and SinR binding sites were confirmed by digestions with SmaI and SspI, respectively, as these sites were introduced in the primers to create the mutations. pS_HZ and pS_RZ were introduced into *B. subtilis* 168 to give 168-S_HZ and 168-S_RZ, and the promoter activity was compared with those of 168-SZ and 168-HZ. The β-galactosidase activities in the three strains 168-S_HZ, 168-

TABLE 2. Primers used in this study

Primer	Sequence (5′ to 3′)[a]	Restriction site(s)[b]
KKR28	GGTAAAGCTT**AGGATATCCGAGC**	HindIII then EcoRV
KKR36	CTTAGGATCC**ATGATTCATCTCC**	BamHI
KKR67	CGTAAGCTT**AATCAGAAGACGCGC**	HindIII
KKR103	CGACTGCAG**CGGCATGGTCAGGAT**	PstI
KKR127	**CGCTTTGCGTGACGGATTATC**	
KKR215	GCACCCGGG**CAGCGGCATGGTCA**	SmaI
KKR216	GCACCCGGG**ATCGGGTCTGTCTTC**	SmaI
KKR217	ATTTAT**AACTGCCCATGAAATCAGC**	SspI
KKR218	ATTAAAA**GTGGGCCGATAAGGTC**	SspI
KKR253	GCAGCTAGC**AGCTCGTTAAAATCAAG**	NheI
KKR254	CGAGCTAGC**TTCCGGGGTATGCTG**	NheI

[a] Nucleotides in boldface are complementary to the genome sequence.
[b] Underlined in the corresponding sequence.

TABLE 1. Plasmids and strains used in this study

Plasmid or strain	Description or genotype[a]	Source or reference[b]
Plasmids		
pRB381	*E. coli-Bacillus* shuttle vector for translational fusion with β-galactosidase gene; *'lacZ* Km^r Ap^r	BGSC (4)
pPZ	pRB381 bearing 850-bp insert (−817 to +33) containing ScoC and SinR binding sites, *epr* promoter, RBS, and ATG in translation fusion with *lacZ* gene	Laboratory stock
pSZ	pRB381 bearing 455-bp insert (−422 to +33) containing ScoC and SinR binding sites, *epr* promoter, RBS, and ATG in translation fusion with *lacZ* gene	This study
pHZ	pRB381 bearing 341-bp insert (−312 to +33) containing *epr* promoter, RBS, and ATG in translation fusion with *lacZ* gene	This study
pS_HZ	pSZ with mutated ScoC binding site	This study
pS_RZ	pSZ with mutated SinR binding site	This study
pS200Z	pSZ with 200-bp DNA insertion between ScoC and SinR binding sites	This study
Strains		
168	*trpC2*	BGSC
168-SZ	*B. subtilis* 168 bearing pSZ	This study
168-HZ	*B. subtilis* 168 bearing pHZ	This study
168ΔH	*trpC2 scoC*::Sp^r	Laboratory stock
168ΔR	*trpC2 sinR*::Cm^r	Laboratory stock
168ΔH-SZ	*B. subtilis* 168ΔH bearing pSZ	This study
168ΔR-SZ	*B. subtilis* 168ΔR bearing pSZ	This study
168-S_HZ	*B. subtilis* 168 bearing pS_HZ	This study
168-S_RZ	*B. subtilis* 168 bearing pS_RZ	This study
168-S200Z	*B. subtilis* 168 bearing pS200Z	This study

[a] Km^r, kanamycin resistance; Ap^r, ampicillin resistance; Sp^r, spectinomycin resistance; Cm^r, chloramphenicol resistance.
[b] BGSC, *Bacillus* Genetic Stock Center.

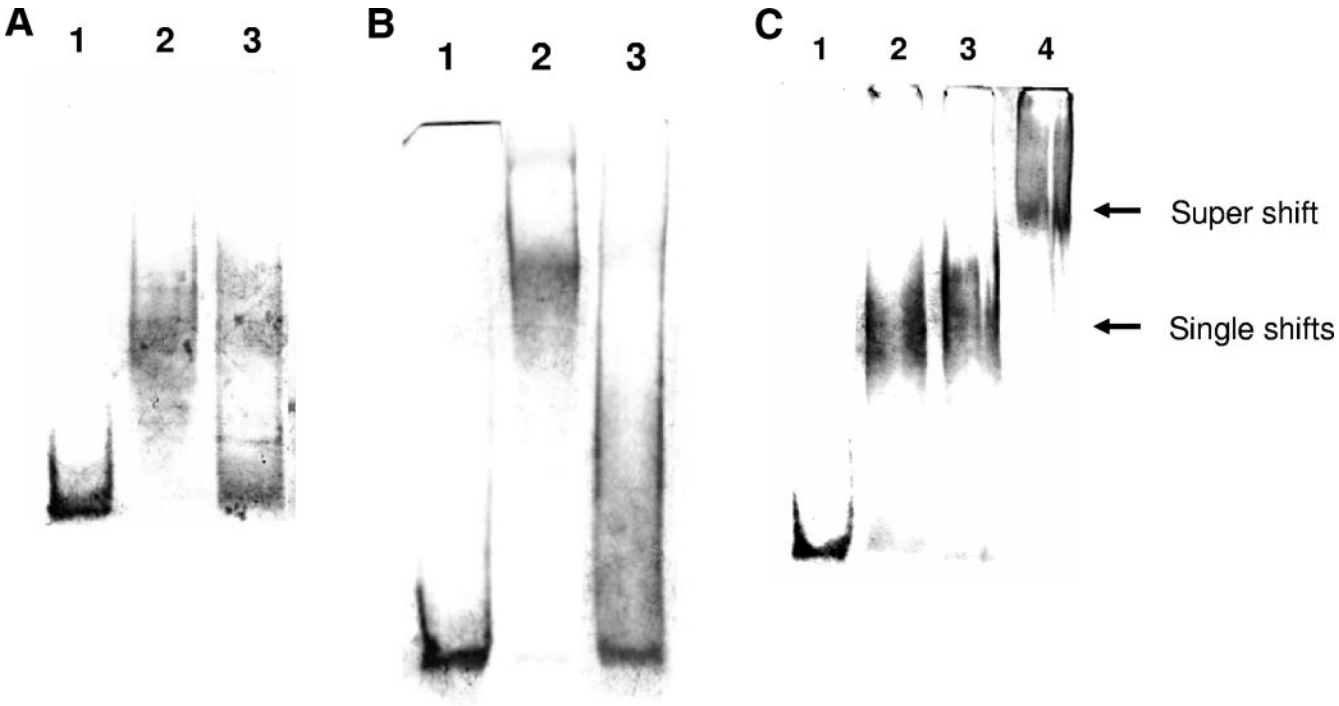

FIG. 2. (A) EMSA with ScoC. Binding reactions were carried out with 10 nM DIG-labeled *epr* probe (~150 bp) and ScoC (1 μM) in a 20-μl reaction buffer containing 20 mM HEPES, pH 7.6, 1 mM EDTA, 10 mM $(NH_4)_2SO_4$, 1 mM dithiothreitol, Tween 20 (0.2% [wt/vol]), 30 mM KCl, 1 μg poly(dI-dC), and 0.1 μg poly-L-lysine at 37°C for 15 min. The bound product was electrophoresed on a 5% polyacrylamide gel in 0.25× Tris-borate-EDTA buffer at 4°C and electroblotted onto Nylon membrane, and DNA was detected with NBT/BCIP (Roche Applied Science; www.roche-applied-science.com/pack-insert/3353591a.pdf). Lane 1, DIG-labeled *epr* probe; lane 2, *epr* probe plus ScoC; and lane 3, *epr* probe plus ScoC plus 100× molar excess unlabeled probe. (B) EMSA with SinR (12 μM). (The binding conditions, electrophoresis, and detection method are as described for panel A. Lane 1, DIG-labeled *epr* probe (~150 bp, 10 nM); lane 2, *epr* probe plus SinR; and lane 3, *epr* probe plus SinR plus 100× molar excess unlabeled probe. (C) EMSA with ScoC (1 μM) and SinR (12 μM). (The binding conditions, electrophoresis, and detection method are as described for panel A). Lane 1, DIG-labeled *epr* probe (~150 bp, 10 nM); lane 2, *epr* probe plus ScoC; lane 3, *epr* probe plus SinR; and lane 4, *epr* probe plus ScoC plus SinR.

S_RZ, and 168-HZ were very similar (3,450, 3,550, and 3,500 Miller units, respectively), as compared to 200 Miller units in 168-SZ. The results show that the ScoC and SinR binding sites are important for negative regulation of *epr* and that mutation of either of them completely relieves repression. The observation of an "all-or-none" repression once again emphasizes the requirement of both proteins for repression. Figure 2C shows that both proteins are capable of binding to the *epr* promoter, as evidenced by a supershift in retardation in the EMSA (lane 4) compared to ScoC or SinR alone (lanes 2 and 3, respectively), indicating that they do not affect each other's binding. Furthermore, mutation of either the ScoC or SinR binding site eliminated the binding of their specific repressor but not the other (data not shown). Taken together, our results suggest that binding of the two repressors to their respective sites could result in a synergistic interaction between the two proteins and that the distance between the two sites could be critical for their interaction. The distance between the ScoC and SinR binding sites is 62 bp. If the distance between the two sites were increased, then repression by ScoC and SinR might be abolished. To determine if this was the case, the promoter activity in a construct, pS200Z (Table 1), in which the distance between the two sites was separated by an additional 200 bp was compared with that of pSZ in *B. subtilis* 168. pS200Z was constructed by PCR amplification of two products obtained from pPZ with primers KKR67/KKR253 and KKR254/KKR36 (Table 2) and cloned sequentially into pBR322 at HindIII/NheI and NheI/BamHI, respectively. A 200-bp DNA fragment was derived from plasmid pET3a by EcoRV digestion and inserted between the ScoC and SinR binding sites within the pBR322 recombinant that was restricted with NheI and filled in with Klenow enzyme. The *epr* promoter segment was reamplified with primers KKR28/KKR36 and cloned in pRB381 to give pS200Z, which was introduced into *B. subtilis* 168 to give 168-S200Z. Whereas the β-galactosidase activity in 168-SZ was 200 Miller units, the activity in 168-S200Z was 3,300 Miller units, comparable to that of 168-HZ (3,500 Miller units), showing the dependence on distance for repression by ScoC and SinR and thus suggesting the interaction of the two proteins for corepression. Insertion of the 200-bp DNA does not, however, affect the binding of the two proteins, as evidenced by the observation of a supershift in the presence of the two proteins (data not shown).

Corepression by ScoC and SinR has not been previously reported in *B. subtilis*, although the capability of these two proteins to interact has been demonstrated in a LexA-based bacterial two-hybrid system (30). In fact, there appear to be only a few examples of corepression described in both prokaryotes and eukaryotes (7, 23, 24, 38). Several mechanisms have been described for corepression. They may involve direct

contacts between proteins that bind DNA, as observed with CytR and cyclic AMP (cAMP)-cAMP receptor protein (CRP) in *E. coli* (17, 18, 29) and with MecI and BlaI in *Staphylococcus aureus* (24). The interaction between two DNA binding proteins may either require an additional factor to link the two proteins, as in the case of the nuclear protein CBP that links the basal transcription factor TFIIB with CREB (19), or may assist in the bending of DNA, thereby facilitating the interaction of the two proteins. In *E. coli*, bending of DNA by integration host factor (IHF) and HU facilitates interaction between flanking DNA-bound dimers of ParB (10, 13) and GalR (1, 2, 22), respectively. In some instances, one protein may regulate another DNA binding protein, as in the case of bacteriophage P1, in which the Bof protein affects the conformation of C1 and stimulates its binding with DNA (20, 37). In another instance, in phage P1, Doc and Phd autoregulate their own transcription by corepression (23). When only Phd was expressed, partial repression of the operon was observed. However, when both Phd and Doc were coexpressed, there was a dramatic enhancement in repression. In contrast, partial repression of *epr* is not observed with ScoC or SinR alone. Only when both are present does repression of *epr* occurs. It is possible that this system could be used to screen a library of genes whose products interfere with corepression, allowing one to identify proteins that interact with ScoC, SinR, or both proteins.

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