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Direct Regulation of *Bacillus subtilis phoPR* Transcription by Transition State Regulator ScoC▽

Bindiya Kaushal, Salbi Paul, and F. Marion Hulett*

Laboratory for Molecular Biology, Department of Biological Sciences, University of Illinois at Chicago, Chicago, Illinois 60607

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Induction of the Pho response in *Bacillus subtilis* occurs when the P_i concentrations in the growth medium fall below 0.1 mM, a condition which results in slowed cellular growth followed by entry into stationary phase. The *phoPR* promoter region contains three σ^A-responsive promoters; only promoter P_{A4} is PhoP autoregulated. Expression of the *phoPR* operon is postexponential, suggesting the possibility of a repressor role for a transition-state-regulatory protein(s). Expression of a *phoPR* promoter-*lacZ* fusion in a *scoC* loss-of-function mutant strain grown in low-phosphate defined medium was significantly higher than expression in the wild-type strain during exponential growth or stationary phase. Derepression in the *scoC* strain from a *phoP* promoter fusion containing a mutation in the CcpA binding site (*cre1*) was further elevated approximately 1.4-fold, indicating that the repressor effects of ScoC and CcpA on *phoP* expression were cumulative. DNase I footprinting showed protection of putative binding sites by ScoC, which included the −10 and/or −35 elements of five (P_{B1}, P_{E2}, P_{A3}, P_{A4}, and P_{A6}) of the six promoters within the *phoPR* promoter region. P_{A6} was expressed *in vivo* from the *phoP cre1* promoter fusion in both wild-type and *scoC* strains. Evidence for ScoC repression *in vivo* was shown by primer extension for P_{A4} and P_{A3} from the wild-type promoter and for P_{A4} and P_{E2} from the *phoP cre1* promoter. The latter may reflect ScoC repression of sporulation that indirectly affects *phoPR* transcription. ScoC was shown to repress P_{A6}, P_{A4}, P_{E2}, and P_{B1} *in vitro*.

The majority of *Bacillus subtilis* genes, which are induced in response to phosphate-limiting growth conditions, are controlled by one of two major global regulatory systems, the PhoP-PhoR two-component signal transduction (TCS) or SigB, a stress sigma factor that is activated in response to limiting P_i. An unknown regulatory system may also exist since a few genes identified as P_i starvation-induced do not depend on either PhoP-PhoR or SigB (3).

Phosphorylated PhoP (PhoP~P) is required for activation or repression of Pho regulon genes. During activation, PhoP binds to a core binding region located between −20 and −60 (relative to the translation start site) on the coding strand, which consists of four repeats of a 6-bp consensus sequence, TT(A/C/T)A(C/T)A, separated by four to six nonconserved base pairs (11, 12). Activated promoters may have additional binding sites either 5′ (11, 12) of the core binding region or 3′ (25) within the coding region that are required for full promoter activity. Activated promoters have no −35 consensus and require PhoP~P for activation (33). The exception is that during autoinduction of the *phoPR* promoter, PhoP~P enhances activity of promoters that have low-level activity without PhoP~P (27). At repressed promoters there are usually two consensus repeats on the noncoding strand upstream of the transcription start site, and PhoP oligomerizes along the DNA into the coding region (19).

The characterization of the *B. subtilis* phosphate deficiency response controlled by PhoPR has revealed that regulatory networks involving multiple two-component systems function in an interdependent manner to make the best use of environmental conditions at the time. The PhoP-PhoR TCS is part of a signal transduction network that consists of at least three TCSs (PhoP-PhoR, ResD-ResE, and Spo0A~P) and a transition state regulator, AbrB (19, 41). The Pho response is positively activated upstream of PhoPR via two parallel pathways involving the ResD-ResE TCS and AbrB (41). An *abrB* mutation causes a slight reduction in the Pho regulon response (18, 21) while a deletion mutation in *resD* leads to an 80% reduction in Pho regulon gene expression (41). AbrB was shown to be essential for the 20% remaining Pho regulon expression in an *resD* mutant strain when an *abrB resD* double mutant (41) showed no Pho regulon gene induction.

Characterization of ResDE TCS regulon genes has shown that ResD plays an indirect role in Pho regulon induction via heme A synthesis required for terminal oxidases (aa_3 and caa_3) (27, 42) that oxidize reduced quinones. Reduced quinones were shown to inhibit autophosphorylation of the PhoR *in vitro*, suggesting that it was the ResD role in terminal oxidase production that positively modulates the PhoR signal (35) upstream of PhoPR. Consistent with this idea, *resD* mutants containing a spontaneous mutation in *rex* (formerly *ydiH*), a repressor of *cydABCD* encoding *bd* oxidase (34), allowed expression of *cydABCD* during Pho induction, which bypassed the requirement for ResD for full Pho induction (35). Together, these data indicate that the terminal oxidase *bd*, encoded by *cydABCD*, was sufficient to replace the loss of caa_3 and aa_3 in the *resD* mutant strain by restoring the terminal oxidase function of oxidation of reduced quinones that inhibit PhoR autophosphorylation. Spo0A~P, produced by the Spo0A phosphorelay system (6), represses the Pho response by negatively regulating *abrB* expression (37) and *resDE* expression via the *resA* promoter (M. Hulett and G. Sun, un-

* Corresponding author. Mailing address: Laboratory for Molecular Biology, Department of Biological Sciences, University of Illinois at Chicago, 900 S. Ashland Avenue (M/C 567), Chicago, IL 60607. Phone: (312) 996-5460. Fax: (312) 413-2691. E-mail: Hulett@uic.edu.
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TABLE 1. Characteristics of each promoter identified within the *phoPR* operon promoter region

phoPR promoter	Position[a]	σ Factor[b]	Activator	Repressor
P_{A6}	−175	σ^A		CcpA
P_5	−93	Unknown[c]	Unknown[d]	Unknown
P_{A4}	−69	σ^A	PhoP∼P	
P_{A3}	−48 to −49	σ^A	Unknown[e]	
P_{E2}	−36 to −38	σ^E	PhoP∼P	
P_{B1}	−23	σ^B		

[a] Relative to the A, position +1, of the translation start site, ATG.
[b] Identified by *in vitro* transcription using core RNAP with purified sigma factor (27, 32).
[c] Not $E\sigma^A$, $E\sigma^B$, $E\sigma^E$, or $E\sigma^H$ responsive (Paul and Hulett, unpublished data).
[d] Transcript observed in only a σ^B mutant (27, 32).
[e] Missing or poor −35 element; very weak promoter *in vitro* compared to *in vivo*; probably missing activator(s) *in vitro* (27, 32).

published data). Positive regulation of Pho regulon gene expression via AbrB is not well understood.

The complexity of the *phoPR* operon promoter region has become apparent over the past decade, revealing a very versatile promoter. Expression from the *phoPR* promoter represents the sum of the six promoters (Table 1), with each responding to specific growth phase and environmental signals (27, 32). Several forms of RNA polymerase (RNAP) holoenzymes are required for transcription of the *phoPR* operon: three $E\sigma^A$-responsive promoters (P_{A3}, P_{A4}, and P_{A6}), one $E\sigma^B$ promoter (P_{B1}), and one $E\sigma^E$ promoter (P_{E2}) (Table 1). The form of RNAP required for the P_5 promoter remains unknown. P_{A4} is largely responsible for low-level transcription from the *phoPR* promoter during exponential growth in low-phosphate defined medium (LPDM) before autoinduction. P_{E2} is expressed during stationary phase but not in a *sigE* mutant strain (27, 30). Autoinduction by PhoP∼P enhances transcription from P_{A4} and P_{E2} (27). P_5 was induced only in a *sigB* mutant strain, perhaps in response to increased P_i deficiency stress caused by the absence of SigB-regulated phosphate starvation-induced (PSI) genes (19, 27, 30). P_{A6} was expressed in a *ccpA* mutant strain (32). P_{A3} is more strongly induced *in vivo* than *in vitro*, probably because it lacks an unknown activator required to compensate for its poor −35 consensus (27). To date, we have accumulated data that uncover layers of regulation placed on the Pho response by exploring the ResDE TCS, CcpA, and Spo0A∼P (19, 27, 32, 35), but how transition state regulators affect the Pho response in *B. subtilis* during transition from late exponential growth to the early stationary phase (when P_i concentration is <0.1 mM) (21) remains a question. AbrB binds extensively to the *phoPR* operon promoter region (M. A. Strauch and F. M. Hulett, unpublished data).

The transition state of *B. subtilis* has been characterized by the expression of functions that are not expressed during exponential growth but initiate expression as cells enter the stationary growth phase. Transition phase functions include production of antibiotics, synthesis of flagella, development of competence for DNA uptake, motility, and production of degradative enzymes, including alkaline phosphatases, that have been shown to be regulated by transition state regulators (28, 29, 38–40). Among the best-studied *B. subtilis* transition state regulators are AbrB, ScoC, and Sin.

ScoC was first identified as a sporulation control locus (4) and has also been referred to as *hpr* (17) or *cat* (15). ScoC is a negative regulator of extracellular proteases (*nprE* and *aprE*), *sinI*, and both oligopeptide permeases, *app* and *opp*, either of which is sufficient to supply the essential oligopeptide permease function for sporulation initiation (23). ScoC is a MarR-type regulator whose transcription is activated by AbrB and has been reported to repress alkaline phosphatases (4, 7). A consensus DNA binding sequence, RATANTATY, was shown by footprint analysis to lie upstream of the *nprE*, *aprE*, and *sinI* genes (22). More recently, it was shown that in the presence of 2% glucose, *scoC* does not require functional AbrB for its expression (36). These observations suggest that AbrB and ScoC might independently regulate the *phoPR* operon by affecting the expression of one or more transcription start sites.

Several observations led us to explore the possibility of a role for ScoC in *phoPR* expression: (i) the presence of three σ^A transcription start sites in the *phoPR* promoter region although most *phoPR* expression is postexponential, (ii) the presence of two putative consensus ScoC binding sites within the *phoPR* promoter region that contain an 8/9-bp match to the ScoC binding consensus (RATANTATY) (22), and (iii) the occurrence of Pho induction in the medium when the P_i concentrations fall below 0.1 mM during the transition state of the cells.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains, plasmids, and primers used in this study are described in Tables 2 and 3. To construct MH7415, JH642 was transformed with linearized plasmid pJM2501 (28), and the transformants were selected for chloramphenicol (Cm) resistance (5 μg/ml) on tryptose blood agar base (Difco), containing 0.5% glucose. To construct MH7416, MH7415 (Cm^r) was transformed with linearized pJL62 (1.1-kb spectinomycin cassette cloned into NcoI site of pJH101) (14), and transformants were selected for spectinomycin (Spc) resistance (100 μg/ml) and screened for Cm sensitivity. To construct MH7400, an isogenic *scoC* mutant strain containing a *phoPR-lacZ* fusion in the *amy* locus, MH5562, was transformed with chromosomal DNA from MH7416, and transformants were selected for Spc resistance (100 μg/ml). MH7417 was constructed via a single crossover through homologous recombination of the *phoP cre1-lacZ* fusion in pMUTIN2 (pAT14 [32]) into the *scoC* mutant, MH7416, selecting for erythromycin resistance (5 μg/ml).

Growth media and enzyme assays. For the expression of *phoPR-lacZ* fusions in the wild-type (WT) and *scoC* mutant strains, the cells were cultured in LPDM containing 2% glucose (LPDMG) as the carbon source as described previously (20). Isogenic *scoC* mutant strains exhibited a very severe growth defect when grown for expression of the *phoPR-lacZ* fusions in LPDMG. Because microarray studies (7) indicated that *scoC* mutations affect the synthesis of isoleucine, arginine, and valine, addition of these amino acids (0.5 mg/ml) along with routinely added amino acids in LPDMG overcame the growth defect and allowed us to study the effect of *scoC* mutations on *phoPR* expression under phosphate starvation conditions. β-Galactosidase specific activity (U/mg protein) was determined as described previously (13). The assay was performed at 37°C. The activity unit was defined as 0.33 nmol of *ortho*-nitrophenol produced per minute.

Overexpression and purification of ScoC. *Escherichia coli* DH5α was used as a host for plasmid construction. *E. coli* BL21(DE3) pLysS (Novagen) served as host for overexpressing the ScoC protein. The *scoC* gene was amplified from *B. subtilis* JH642 chromosomal DNA by PCR using primers FMH1011 at the 5′ end of the gene and FMH1012 at the 3′ end of the gene. The PCR product was cloned into pCR2.1 (Invitrogen) to construct pBK6.1. The *scoC* gene was then released from pBK6.1 by XhoI and BamHI digestion and subcloned into the XhoI and BamHI sites of pET16b (Novagen), generating pBK6. The *scoC* gene was confirmed by sequencing. The pBK6 plasmid contains a T7 *lac* promoter, the codons for 10 histidine residues, and an engineered factor Xa site upstream of the *scoC* gene. BL21(DE3) pLysS cells containing pBK6 were grown in Luria Bertani medium (100 ml) containing carbenicillin (50 μg/ml) and ampicillin (100 μg/ml) at 37°C overnight and were inoculated into 2 liters of the same medium at a ratio of 1 to 100. The cells were grown at 30°C until the optical density at 540 nm (OD_{540}) was 0.6; isopropyl-β-D-thiogalactopyranoside (IPTG; 1 mM) was

TABLE 2. Bacterial strains and plasmids

Strain or plasmid	Genotype or description	Source or reference
E. coli strains		
DH5α		Lab stock
BL21(DE3) pLysS		Novagen
B. subtilis strains		
JH642	*pheA1 trpC2*	J. A. Hoch
MH5562	*pheA1 trpC2 amy*::*pho-lacZ* Cm^r	27
MH7415	*pheA1 trpC2 scoC*::(pJM2501) Cm^r	This work
MH7416	*pheA1 trpC2 scoC*::*(pJM2501)* Cm^s::pJL62 Spc^r	This work
MH7400	*pheA1 trpC2 scoC*::(pJM2501) Cm^s::pJL62 Spc^r *amy*::*phoPR-lac Z* Cm^r	This work
MH7417	*pheA1 trpC2 scoC*::(pJM2501) Cm^s::pJL62 Spc^r pAT14Ω *phoP cre1-lacZ* Erm^r	This work
MH6040	*pheA1 trpC2* pAT14Ω *phoP cre1-lacZ* Erm^r	32
MH6024	*pheA1 trpC2* pAT3Ω *phoP-lacZ* Erm^r	32
Plasmids		
pJM2501	A pUC19 plasmid carrying a 900-bp PvuII fragment containing the *scoC* gene with *cat* gene insertion mutation at PvuI	28
pJL62	1.1-Kb spectinomycin resistance cassette cloned into NcoI site of pJH101; Amp^r Spc^r Tet^r	I. Smith
PCR2.1	A linearized vector having single 3′ deoxythymidine (T) residues; Amp^r Kan^r	Invitrogen
pHT4-*phoPR*	pHT315::Sau3A1 fragment of *B. subtilis* chromosome containing 3′ region of *mdh*, *phoP*, and *phoR* and 5′ *polA*	8; T. Masdek
pBK1	1.364-Kb SmaI-Pau I insert from pHT4-*phoPR* cloned into pUC18	26; this work
pBK6.1	PCR-amplified *scoC* gene (636 bp) from JH642 chromosomal DNA ligated into PCR2.1 vector	This work
pBK6	Vector for overexpression of ScoC; XhoI-BamHI fragment from pBK6.1 encoding *scoC* gene cloned into pET16b at the same restriction sites	This work
pAT3	Full-length *phoPR* promoter from pES2 in pMUTIN2; bp −705 to +92 relative to *phoP* translation start site	32
pAT14	*phoP cre1* promoter from pAT12 in pMUTIN2; Erm^r	32
pAT12	*phoP cre1* promoter in pCR2.1	32
pSB5	*phoPR* promoter in PCR2.1; Amp^r Kan^r; bp −301 to +92 relative to *phoP* translation start site	27
pSB40	396-bp BamHI/EcoRI fragment from pSB5 subcloned into pDH32; Amp^r Cm^r	27

added to the culture, and the cells were collected by centrifugation at 8,000 × *g* for 45 min after a 3-h incubation period. The pellet was resuspended in 30 ml of sonication buffer (50 mM Tris-HCl [pH 8.0], 500 mM NaCl, 5 mM $MgCl_2$, and 20% glycerol); after 1 mM phenylmethylsulfonyl fluoride (PMSF) was added, the cells were sonicated immediately and centrifuged at 16,000 × *g* for 1 h at 4°C. The supernatant fraction was filtered through 0.45-μm-pore-size membrane and applied to a 2.5-ml nickel-nitrilotriacetic acid (Ni-NTA) agarose (Qiagen) affinity column (the Ni-NTA resin was previously equilibrated with sonication buffer in a 1.0-cm by 1.0-cm Econo-column [Bio-Rad]). This column was incubated at 4°C on a rotoshaker for 1.5 h. The column was sequentially washed with the sonication buffer (20 times, 2.5 ml each time) until the OD_{280} of the elute was less than 0.03; it was then washed with 30 mM imidazole in sonication buffer (two times, 2.5 ml each time) at 4°C. The bound protein was eluted with a four-step imidazole gradient from 100 to 400 mM (2.5 ml each of 100 mM, 200 mM, 300 mM, and 400 mM imidazole in the sonication buffer at 4°C). The eluted protein (300 mM and 400 mM imidazole) was dialyzed overnight against sonication buffer at 4°C to remove the imidazole. The protein concentration was determined using a Bio-Rad protein assay (Bio-Rad Laboratories), with bovine serum albumin (BSA) as the standard. The protein was aliquoted and stored at −70°C.

DNase I footprinting. DNase I footprinting experiments were performed as described previously (24). The *phoPR* promoter was amplified from pBK1 in two different reactions to make two fragments, each containing a putative ScoC binding site(s). To assess DNase I protection by ScoC on the 5′ promoter region containing a single putative binding site, 5′-$^{-227}$GAAAGTATT^{-219}-3′ (see Fig. 1), on the coding strand, a 232-bp-long probe for the coding strand was prepared by amplifying the *phoPR* promoter from pBK1 using radiolabeled primer

TABLE 3. Primers

Primer	Sequence (restriction enzyme)[a]	Function
FMH1011	5′-GACTCGAGATGAATCGAGTGGAACCGCCC-3′ (XhoI)	Forward primer for the amplification of *scoC* gene
FMH1012	5′-GCGGATCCCATCATGAAGCATTTTGATTA-3′ (BamHI)	Reverse primer for the amplification of *scoC* gene
FMH880	5′-GAATTC^{-326}GTAGGCGGCAACGG^{-313}-3′ (EcoRI)	Forward primer for the amplification of the 5′ *phoPR* promoter region
FMH881	5′-$^{-100}$CGACAATTCGCCTTTTACA^{-118}-3′	Reverse primer for the amplification of the 5′ promoter region
FMH1018	5′-GCGGCCGC^{-120}GATGTAAAAGGCGAATTGTCGG^{-99}-3′ (NotI)	Forward primer for the amplification of the 3′ *phoPR* promoter region
FMH1019	5′-CATATG^{+24}CACAACTAAAATTTTCTTGTTC^{+3}-3′ (NdeI)	Reverse primer for the amplification of the 3′ *phoPR* promoter region
FMH1025	5′-ATATAAAAGCATTAGTGTATCAATTCAAGC-3′	Primer within *lacZ* fusions used for primer extension analysis

[a] Superscript base pair numbering is relative to the A of the ATG translation start site of *phoP*. Restriction sites are underlined.

FMH880 and nonradiolabeled primer FMH881. For the noncoding strand preparation, a PCR was set up using nonradiolabeled primer FMH880 and radiolabeled primer FMH881. PCR products were gel extracted using a Qiagen gel extraction kit. To assess the DNase I protection by ScoC on the 3′ promoter region containing a single putative binding site, $^{-78}$AATAAAATC^{-71} (see Fig. 1), on the coding strand, a 156-bp-long probe for the coding strand was prepared by amplifying the *phoPR* promoter from pBK1 using radiolabeled primer FMH1018 and nonradiolabeled primer FMH1019. For the noncoding strand, a PCR was set up using nonradiolabeled primer FMH1018 and radiolabeled primer FMH1019.

In each reaction mixture, the ScoC protein at increasing concentrations of 0.0, 0.3, 0.5, 1.0, 2.0, 3.0, 4.0, and 5.0 μM was incubated with the probe at a 50 nM concentration in the binding buffer (10 mM Tris-HCl [pH 7.5], 1 mM EDTA [pH 7.5], 50 mM KCl, 10% glycerol, and 1 mM dithiothreitol) at room temperature and digested with 2 μl of DNase I (1 U/μl) for 1 min for protein-containing samples and for 30 s for protein-free samples. The reaction was stopped, and the DNA fragments were purified by phenol-chloroform extraction, followed by ethanol precipitation. The DNA fragments were run on a 6% polyacrylamide gel containing 7 M Urea and detected by using a Storm 860 PhosphorImager (Molecular Dynamics) or X-ray film.

RNA preparation and primer extension analysis. Total RNA was isolated, by using an RNeasy Midi Kit (Qiagen), from MH5562, MH7400, MH6040, and MH7417 strains grown in LPDMG at various time intervals (exponential stage, transition stage, and postexponential growth) during a 12-h growth period. Two volumes of the RNA Later stabilization reagent (Qiagen) were added to 1 volume of the cell culture; they were mixed by vortexing and kept at room temperature for 10 min. Cells were immediately collected by centrifugation at 5,000 rpm at 4°C for 10 min. This pellet was then used to isolate total RNA using the kit according to the manufacturer's instructions. Purified RNA was stored at −80°C until use. A primer specific for *lacZ* was used for primer extension analysis of RNA. FMH1025 was end labeled for 30 min at 37°C in a 50-μl 1× T4 polynucleotide kinase (PNK) forward reaction buffer (MBI Fermentas) containing 150 μCi [γ-^{32}P]ATP (6,000 Ci/mmol or 10 mCi/ml; Perkin-Elmer) and 50 units of PNK (10 U/μl). The labeling reaction was stopped by heating the mixture at 90°C for 10 min. A total of 50 μg of total RNA was used in each primer extension reaction mixture. A primer extension procedure previously described (9) was used with some modifications. The end-labeled primer was annealed to 50 μg of total RNA by mixing and precipitating it together with 0.1 volume of 3 M sodium acetate and 2.5 volumes of 100% ethanol; the sample was chilled on dry ice for 30 min and centrifuged at 12,000 rpm for 10 min; the pellet was air dried, suspended in 25 μl of 1× reverse transcriptase buffer (RTB) (MBI Fermentas), and incubated in a preheated water bath at 80°C. The water bath was allowed to cool at room temperature until it reached 37°C. Two microliters of RNase inhibitor (MBI Fermentas) was added, and incubation continued at 37°C for 16 h. To each reaction mixture, 25 μl of the primer extension mix (1× RTB, 10 mM each deoxynucleoside triphosphate [dNTP], 1 U of avian myeloblastosis virus [AMV] reverse transcriptase, 0.3 units of RNase inhibitor [MBI Fermentas]) was added, and samples were incubated in a water bath at 42°C for 1 h. Reactions were stopped by incubation at 90°C for 15 min. Primer extension products were precipitated with 0.1 volume of 3 M sodium acetate and 2.5 volumes of 100% ethanol at −80°C for 30 min; they were collected by centrifugation (12,000 rpm) at 4°C, and the pellets were air dried. The pellets were suspended in 8 μl of Tris-EDTA (TE) buffer, pH 8.0, and 4 μl of a sequencing dye (98% formamide, 10 mM EDTA, 0.1% xylene cyanol, 0.1% bromophenol blue). Samples were heated at 85°C for 5 min and run on a preheated 5% sequencing gel. A sequencing ladder was made by annealing the same end-labeled primer, FMH1025, to pSB40 or pAT14 for experiments shown in Fig. 4 and 6, respectively, using a Sequenase, version 2.0, sequencing kit (U.S. Biochemicals Corp.) according to the manufacturer's instructions.

RESULTS

ScoC represses the transcription of the *phoPR* promoter. The *phoPR* operon promoter region contains three vegetative σ^A promoters, which raised the question of why the expression of this operon is so low during exponential growth and induced principally postexponentially. Part of the answer is that PhoP∼P, which is present only after a culture experiences growth-limiting P_i concentrations (0.1 mM), enhances expression of one of the three σ^A promoters, P_{A4}. Transition state regulators, which silence promoters during exponential growth that would otherwise be active, are also candidates for *phoPR* repression.

The *phoPR* promoter contains two putative consensus DNA binding sites for ScoC with eight of nine residues matched to the ScoC DNA binding consensus sequence RATANTATY (22) shown in Fig. 1. The 5′ promoter region contains a putative binding site, $^{-227}$GAAAGTATT^{-219} (Fig. 1, site A) on the coding strand. Similarly, the 3′ promoter region contains one putative binding site, $^{-78}$AATAAAATC^{-71} (Fig. 1, site B) on the coding strand, with eight of nine residues matched, and two less-conserved ScoC binding sites, $^{-51}$CATAAAATA^{-43} and $^{-23}$AATTATAAT^{-15} (Fig. 1, sites C and D, respectively).

To determine if ScoC had a role in *phoPR* promoter regulation, we grew strain MH5562, which contained a single copy of the *phoPR-lacZ* promoter fusion at the *amyE* locus in JH642, and an isogenic strain, MH7400 (MH5562 with a *scoC* mutation), in LPDMG plus isoleucine, arginine, and valine in addition to routinely added amino acids, over a period of 12 h (Fig. 2). The WT strain, MH5562, exhibited low-level transcription from the *phoPR* promoter fusion during P_i-replete exponential growth (0 to 4 h). As the concentration of P_i decreased (5 h to 12 h) below 0.1 mM (2, 21), there was induction of the transcription of the *phoPR* operon in the WT strain, which is normally observed in JH642 grown in LPDMG (27). The isogenic *scoC* mutant strain, MH7400, had a higher level of *phoPR* transcription (5- to 10-fold) during exponential growth (at 0 to 4 h) than the WT strain (MH5562). After induction in both the strains, the *scoC* mutant maintained *phoPR* expression approximately 2-fold higher than the WT strain. These data suggested that ScoC represses, directly or indirectly, *phoPR* expression throughout growth, albeit to a lesser extent after transition into stationary phase (Fig. 2).

ScoC binds directly to the *phoPR* promoter. The above study revealed higher transcriptional levels in the *scoC* mutant than in the WT strain, leading us to ask if the repressor function of ScoC might be due to the binding of the ScoC protein at the observed putative binding sequences on the *phoPR* promoter. To test this hypothesis, we preformed gel retardation studies and observed evidence that purified ScoC protein could interact with DNA sequences located in the promoter region of the *phoPR* operon (data not shown). To determine the regions of the *phoPR* promoter protected by binding of ScoC protein, DNase I footprinting experiments were performed using purified ScoC and each of two *phoPR* promoter fragments. One fragment was a 226-bp-long probe (bp −326 to bp −100), called the 5′ promoter region, that contained the P_{A6} transcription start site, and the second was a 144-bp-long probe (bp −120 to bp +24), called the 3′ promoter region, that contained five transcription start sites (P_{B1}, P_{E2}, P_{A3}, P_{A4}, and P_5).

Using the 5′ region of the *phoPR* promoter as a probe, ScoC protected the promoter region from −228 to −196 on the coding strand (Fig. 3A) and from −203 to −228 on the noncoding strand (Fig. 3B). The distinct protection by ScoC on the 5′ promoter region shown by footprint analysis encompasses the putative ScoC binding site on both strands (Fig. 1, site A). Using the 3′ *phoPR* promoter region as a probe, ScoC protected the promoter region from −34 to −46 and from −69 to −84 on the coding strand (Fig. 3C). The protected regions on the noncoding strand were from −24 to −31, −34 to −54, and

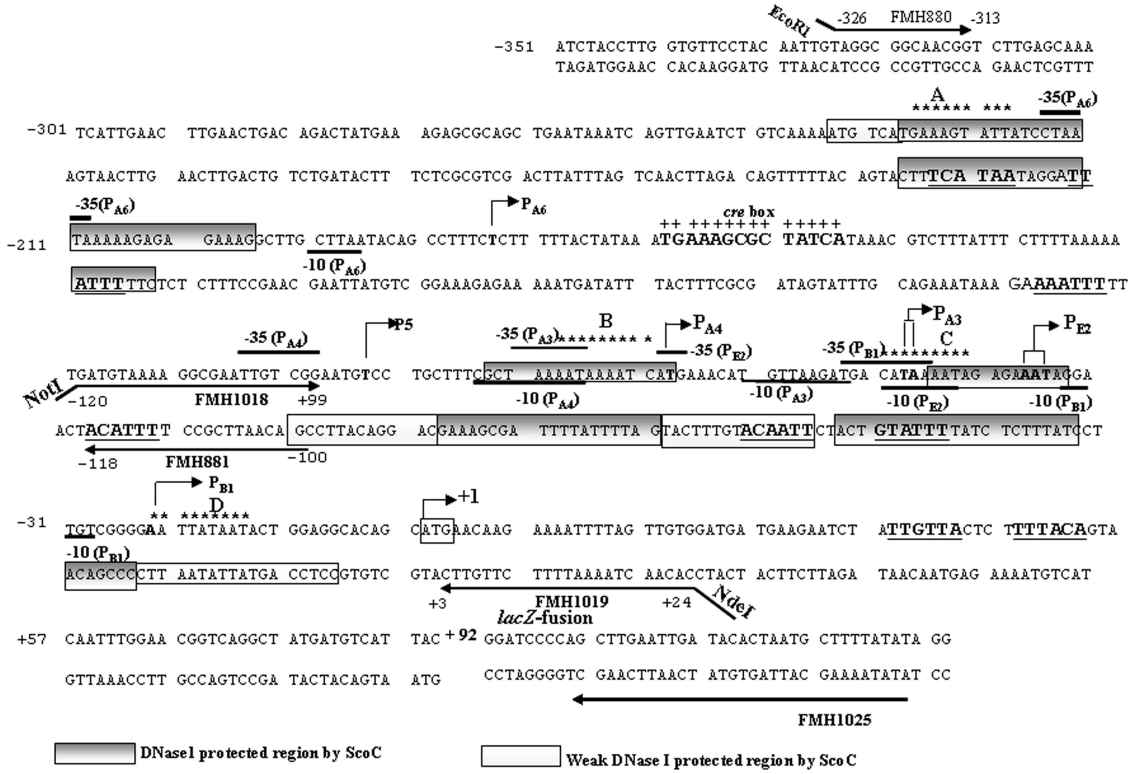

FIG. 1. The *phoPR* promoter sequence and 5′ PhoP coding sequence showing six transcription start sites along with the putative ScoC binding sites. Transcription start sites for P_{B1}, P_{E2}, P_{A3}, P_{A4}, P_5, and P_{A6} are indicated by boldface sequence and are identified by a bent arrow followed by the promoter number and a letter identifying the form of RNAP (where known) required for the transcription. The −10 region is marked below, and the −35 is marked above the sequence for each promoter based on published consensus sequences (16). The region of +1 to +92 is the 5′ PhoP coding sequence followed by the *lacZ* fusion. The translation start site, ATG, is boxed and identified by a bent arrow marked +1. Sequence numbering is relative to the A of ATG as +1. A single putative ScoC binding site in the 5′ region of the *phoPR* promoter, 5′-$^{-227}$GAAAGTA TT^{-219}-3′ (site A), is located on the coding strand. Three conserved putative ScoC binding sites in the 3′ region of the *phoPR* promoter are 5′-^{78}AATAAAATC^{-71}-3′ (site B), 5′-$^{-51}$CATAAAATA^{-43}-3′ (site C), and 5′-$^{-23}$AATTATAAT^{-15}-3′ (site D) located on the coding strand. The ScoC binding consensus sequence is RATANTATY, where R is A or G, Y is C or T, and N is A, G, C, or T. The putative ScoC binding sites are indicated with nine stars above the sequences. The CcpA binding site (*cre* box) is shown in bold, marked above with plus signs. Primers used are shown as underlined or overlined sequences with an arrow in the direction of synthesis. FMH1025 is the primer used for primer extension analysis specific to the *lacZ* fusion. The ScoC DNase I-protected regions are shown as gray boxes on both strands of the *phoPR* promoter. The white boxes show the weaker ScoC protection. The consensus repeats for PhoP dimer binding, TT(A/C/T)A(C/T)A, are underlined, and the sequence is in boldface.

−71 to −89 (Fig. 3D). Weaker ScoC protection on the noncoding strand could be seen to extend from −7 to −24, −56 to −71, and −90 to −101. The location of the DNase I-protected regions on the 3′ *phoPR* promoter region suggested that the second specific ScoC binding sequence (Fig. 1, site B) was an ScoC binding site on the *phoPR* promoter.

Closer inspection of the sequences protected by ScoC binding on the 3′ *phoPR* promoter region identified two other consensus binding sites, albeit less conserved (Fig. 1, sites C and D). The binding activity of the ScoC protein to the *phoPR* promoter in the present study protected the consensus binding sequence RATANTATY, which was shown by footprint analysis to lie upstream of the *nprE*, *aprE*, and *sinI* genes (22). The footprint analysis of the *phoPR* promoter also revealed that ScoC binding protects the −10 elements of the existing transcription start sites P_{B1}, P_{E2}, and P_{A4} and the −35 elements of the P_{B1}, P_{E2}, P_{A3}, and P_{A6} promoters. These results suggest that the putative ScoC binding sites are important for the interaction of ScoC on the *phoPR* promoter to exert its negative effect.

Increased expression of P_{A3} prior to Pho induction and prolonged expression of P_{A4} and P_{A3} during stationary phase appeared to account for increased *phoPR* expression in an *scoC* strain compared to the WT. Primer extension was preformed to determine which *phoPR* promoter(s) accounts for the increased *phoPR* expression in an *scoC* strain (MH7400) compared to the WT strain (MH5562) shown in Fig. 2. Figure 4 shows the results of primer extension analysis using RNA from MH5562 and MH7400 cultures grown in LPDMG that was isolated hourly, starting 2 h prior to (time T_{-2}) and continuing until 5 h after Pho induction (defined as T_0), as determined by APase assays. Transcription of P_{A3} was observed earlier in the *scoC* mutant strain (Fig. 4B T_{-1}) than in the WT strain (Fig. 4A, T_1) suggesting that P_{A3} contributes to the increased levels of total *phoPR* transcript during exponential growth (Fig. 2). Levels of both P_{A4} and P_{A3} transcripts ap-

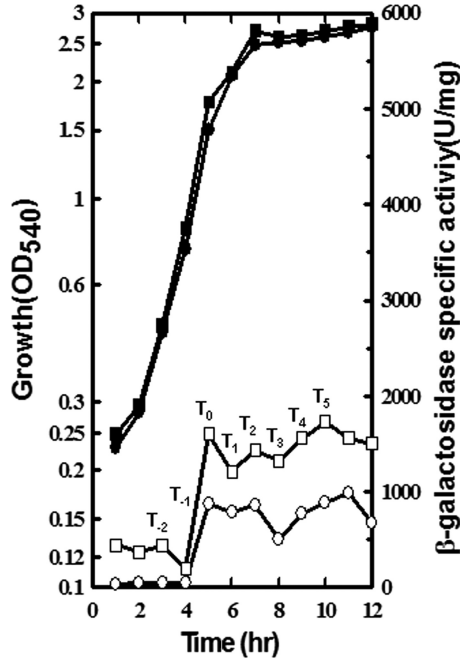

FIG. 2. Effect of *scoC* mutation on *phoPR* transcription throughout growth. Cells were grown in LPDMG over a period of 12 h to monitor the growth and level of *phoPR* transcription. The first hour when APase activity was induced as a reporter for Pho induction is identified as T_0. Solid symbols represent the growth, and open symbols represent the β-galactosidase specific activity of the full-length *phoPR-lacZ* fusion in each strain. Circle, WT (MH5562); square, *scoC* (MH7400).

peared to decrease during stationary phase in the WT strain but not in the *scoC* mutant strain. Levels of P_{E2} appeared higher in the WT than in the *scoC* strain. Together, the last two observations suggest that the sustained expression of P_{A3} and P_{A4} during stationary phase contributed to the higher total *phoPR* expression in the *scoC* strain during that time.

For each primer extension reaction, the radioactivity from each promoter was detected using a PhosphorImager and was quantified using ImageQuant software. The sum of the activity was defined as 100%, and the percent contribution from each promoter was calculated. The data are shown in bar graphs. In the WT samples P_{A4} was the major transcript from T_{-2} to T_0 (60 to 80% of total transcript), but in the *scoC* strain P_{A3} was a major contributor to the total transcript, i.e., nearly 50% of the total transcript by T_{-1}. In the WT strain at T_1, P_{A3} and P_{A4} were nearly equally expressed, with each accounting for 45 to 50% of the total transcript, but thereafter their transcripts decreased in unison between T_2 and T_5, at the same time that P_{E2} was increasing. P_{A3} and P_{A4} each represented less than 20% of total transcript while P_{E2} accounted for over 40% between T_3 and T_5. In the *scoC* strain there was no significant change in P_{A3} and P_{A4} transcripts (T_3 to T_5) such that as P_{E2} transcript increased, the three transcripts became close to 30% each.

P_{B1} transcripts were low throughout growth but increased slightly in both strains during stationary growth. Neither P_5 nor P_{A6} was observed in either strain, as expected.

Maximum expression of the *phoP cre1* promoter fusion in an *scoC* background was greater than in a wild-type strain. A *phoP* promoter fusion in a WT strain which contained a mu-

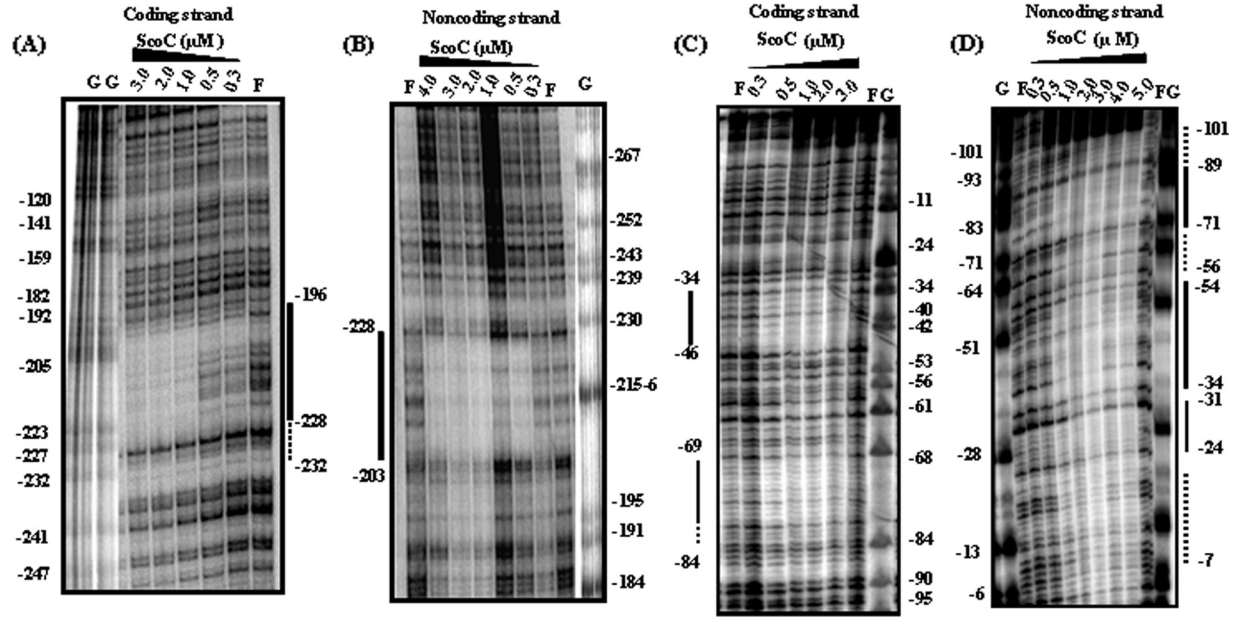

FIG. 3. DNase I footprint analysis of ScoC binding to the *phoPR* promoter. The plasmid pBK1 was used as a template for a PCR probe. The ScoC concentration (μM) is shown at the top of each lane. F, free of ScoC; G, Maxam-Gilbert G sequencing reaction, used as a marker. Base pairs are numbered on coding and noncoding strands relative to the translation start site (as +1). Solid lines identify the DNase I protection. Dotted lines identify the weaker protection by ScoC. (A) DNase I protection by on the coding strand in the 5′ region of the *phoPR* promoter. End-labeled FMH880 and nonlabeled FMH881 were used to create the PCR probe. (B) DNase I protection on the noncoding strand in the 5′ region of the *phoPR* promoter. End-labeled FMH881 and nonlabeled FMH880 were used to create the PCR probe. (C) DNase I protection on the coding strand of the 3′ promoter region. End-labeled FMH1018 and nonlabeled FMH1019 were used to create the PCR probe. (D) DNase I protection on the noncoding strand of the 3′ promoter region. End-labeled FMH1019 and nonlabeled FMH1018 were used to create the PCR probe.

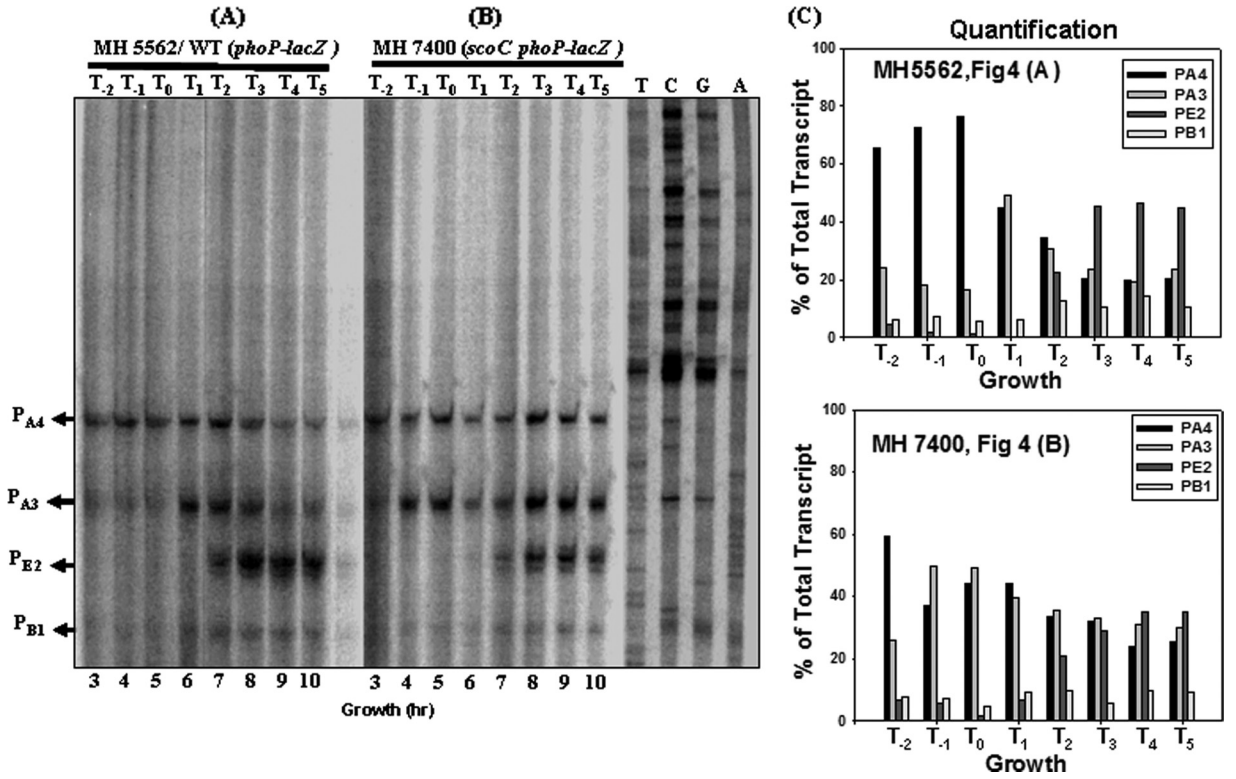

FIG. 4. Primer extension analysis of the *phoPR* promoter region from the total RNA isolated from a WT (MH5562) or a *scoC* strain (MH7400) grown in LPDMG. The end-labeled primer FMH1025 was annealed to RNA isolated from exponential phase, transition stage, or postexponential phase cultures. (A) Lanes 3 to 10 show the primer extension reactions of RNA samples taken from a WT strain (MH5562) growing in LPDMG at the times indicated. The expression from four transcriptional start sites (P_{B1}, P_{E2}, P_{A3}, and P_{A4}) is indicated with arrows. T_0 is the time of Pho induction and T_1, T_2, T_3, T_4, and T_5 are 1, 2, 3, 4, and 5 h of growth, respectively, in LPDMG after Pho induction. T_{-2} and T_{-1} samples were taken at 2 h and 1 h before Pho induction, respectively. (B) Lanes 3 to 10 show the primer extension of RNA samples taken from the *scoC* (MH7400) strain at the times indicated. (C) Quantification of individual transcripts. Radioactivity was determined in arbitrary units for each transcript by phosphorimaging. The sum of all transcripts from a reaction was considered 100%. The percent contribution of each transcript to the total transcription from each primer extension reaction was calculated and plotted in a set of bar graphs. The time of RNA sampling was indicated relative to Pho induction, T_0. Bars correspond to P_{A4}, P_{A3}, P_{E2}, and P_{B1}, respectively.

tation in the CcpA binding site was reported to express a *phoPR-lacZ* fusion at levels similar to those observed in a *ccpA* deletion strain. To ask how ScoC affected P_{A6} transcription, we used strain (MH7417) containing an *scoC* mutation and the *cre1* mutation in the CcpA binding site (*cre* box) of the *phoPR* promoter fusion, which allowed the expression of the *phoP cre1-lacZ* fusion at the *phoP* locus and also retained an intact *phoPR* promoter for *phoPR* operon expression downstream of the plasmid insertion. Isogenic JH642 strains containing a WT *phoPR-lacZ* (MH6024) or a *phoP cre1-lacZ* (MH6040) promoter fusion at the *phoPR* locus were used as controls.

These three strains were grown in LPDMG and assayed for growth and promoter expression over a period of 10 h (Fig. 5). APase induction was measured to determine T_0, the time of Pho regulon induction (data not shown). The strain with the WT *phoPR-lacZ* fusion (MH6024),which exhibited low-level *phoPR* expression under excess phosphate conditions (0 to 4 h), was induced after inorganic phosphate levels became limiting (at 5 to 12 h). Expression from the *phoP cre1-lacZ* fusion in the MH6040 (WT) or MH7417 (*scoC*) strain had considerable *lacZ* activity from the inoculum, as was observed from the same promoter fusion in a *ccpA* mutant that expressed P_{A6} (32). Induction in the *scoC* strain began between hours 2 and 3 (1 h prior to APase induction) and continued to increase until hour 10 h. Induction in the WT strain was delayed until hour 5 and continued until hour 7; thereafter, the specific activity remained stable. The maximum expression from the *phoP cre1-lacZ* fusion in the *scoC* strain was 1.4-fold higher than in the WT strain. The elevated expression of the *phoPR* promoter fusion in MH7417 (*scoC phoP cre1-lacZ*) may be attributed to the derepression of the transcription from the P_{A6} promoter as a result of the *scoC* mutation and the inability of CcpA to bind at the *cre1* site and block the transcription.

A *cre1* mutation in the *phoPR* promoter is sufficient to allow expression of P_{A6}. Primer extension analysis was performed on RNA isolated at various times of growth before and after Pho induction from the MH7417 and MH6040 cultures used in the experiment shown in Fig. 5 to determine if P_{A6} were expressed and which of the six promoters were regulated by ScoC. The data shown in Fig. 6A confirmed the assumption that P_{A6} was expressed from the *phoP cre1-lacZ* fusion in MH6040 (32), which resulted in expression levels similar to those observed in a *ccpA* mutant strain. Also similar to the primer extensions studies using RNA isolated from a *ccpA* strain, weak transcripts from P_5 were observed in either strain with the *phoP cre1* promoter fusion (Fig. 6 A and B).

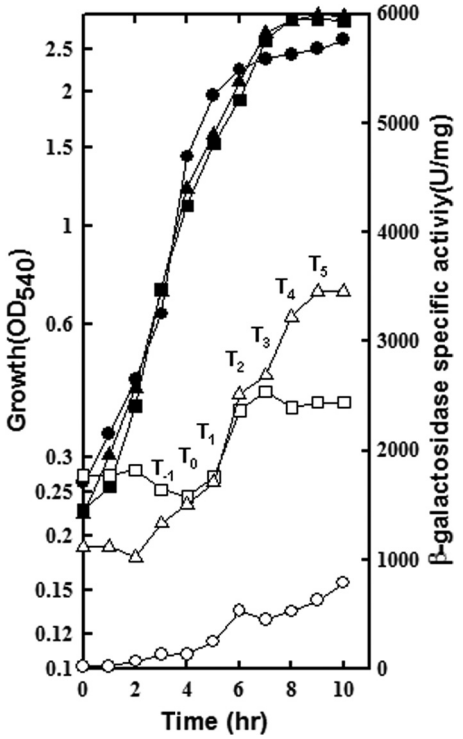

FIG. 5. Combined effect of *scoC* and *phoP cre1* promoter mutation on *phoPR* transcription during growth and stationary phase. Cells were grown in LPDMG for 10 h to monitor growth and level of transcription. Solid symbols represent the growth, and open symbols represent the β-galactosidase specific activity of the full-length *phoPR-lacZ* fusion in each strain. Circle, WT *phoPR-lacZ* (MH6024); square, *phoPR-lacZ cre1-lacZ* strain (MH6040); triangle, *scoC phoPR-lacZ cre1-lacZ* strain (MH7417).

The absence of ScoC may affect *phoPR* promoter transcription directly and indirectly. When individual promoter expression from the *phoP cre1-lacZ* fusion in the *scoC* strain (MH7417) was compared to that in the parent strain (MH6040), a number of changes were noted that may be due to the absence of ScoC (Fig. 6A and B). During the first 3 h (T_{-1} to T_1), P_{A6} was the major transcript in both cultures (≈60% of total transcript) although P_{A4} showed a relative increase in the *scoC* mutant compared to the parent strain, which may be due to the absence of ScoC repression. Two interesting differences in expression were noted at T_2. First P_{E2}, which requires $E\sigma^E$ for expression, was expressed in the *scoC* strain but not in the parent strain, indicating that sporulation initiation had occurred earlier in the *scoC* strain. Further, P_{A6} expression decreased more dramatically in the *scoC* strain than in the parent strain at T_2, consistent with decreasing $E\sigma^A$ concentrations after the initiation of sporulation. Sporulation control, for which *scoC* was named (4), results from ScoC repression of oligopeptide transport expression (*opp* operon) (23), thereby delaying sporulation initiation. The latter two observations discussed here may represent direct roles of ScoC that affect sporulation initiation, which indirectly affects *phoPR* transcription.

Of the three σ^A-responsive promoters, P_{A4} expression was the least affected by the decreasing concentrations of $E\sigma^A$ associated with the initiation of sporulation. This is consistent with *in vitro* transcription studies which indicated that the concentration of $E\sigma^A$ required was decreased with increasing concentrations of PhoP~P at another PhoP~P-enhanced promoter, the *resA* promoter (1).

DISCUSSION

ScoC represses several *phoPR* promoters. Analysis of the data presented here indicated that ScoC downregulates *phoPR* expression during vegetative and postexponential growth. The activity of the *phoPR-lacZ* fusion in the *scoC* loss-of-function mutant strain was elevated 5- to 10-fold during P_i-replete exponential growth compared to activity in the WT strain, in which expression of the *phoPR-lacZ* fusion is barely detectable (Fig. 2). The *phoPR-lacZ* fusion induction, when the cultures entered the stationary phase due to limiting inorganic phosphate, was 2-fold higher in the *scoC* mutant strain than the in the WT strain. *In vitro* transcription studies showed ScoC repression of transcription from P_{A6}, P_{A4}, and P_{B1} transcription start sites using T_0 RNAP and from P_{A4}, P_{A6}, and P_{E2} start sites using T_3 RNAP (data not shown). Our knowledge of promoters P_5 and P_{A3} (Table 1) is insufficient to evaluate them using *in vitro* transcription.

Previous studies have identified an alkaline phosphatase among ScoC-repressed proteins (4, 7). It seems unlikely that ScoC plays a negative role in alkaline phosphatase expression via the *phoPR* derepression reported here. First, increased expression of *phoPR* does not necessarily lead to corresponding changes in *pho* regulon gene expression (10, 31). Second, the conditions under which the *scoC* mutant strains were cultured in the microarray studies (7) were not phosphate limiting. Further, not all APase expression in *B. subtilis* is PhoP~P activated. *phoB* (formerly *phoAIII*) encoding APase B (formerly APase III) (5, 9) is expressed from two differentially regulated promoters, P_S ($E\sigma^E$-responsive promoter) and P_v ($E\sigma^A$-responsive promoter), which are PhoP~P repressed and activated during PSI, respectively (2, 5, 9). The study by Caldwell et al. (7) found a 9.3-fold increase in *phoB* expression in an *scoC* mutant compared to the WT strain at the last time point assayed (310 min after inoculation), an increase which represented the greatest fold increase in a group of sporulation genes dependent on Spo0A and σ^F. This increase could have resulted indirectly from regulation via removal of the repressor role of ScoC in the sporulation process, leading to activation of σ^E and thereby the *phoB* P_s promoter, or directly from the absence of ScoC binding to several putative binding sites observed on the *phoB* promoter, which might account for direct ScoC repression of the upstream *phoB* P_S promoter, or from both.

Negative regulation of the *phoPR* promoter by ScoC and CcpA is cumulative. Another layer of transcriptional regulation on the *phoP* promoter via ScoC was revealed during this study. It was shown previously that CcpA caused repression of the *phoPR* promoter through the P_{A6} transcriptional start site which is positioned upstream of the *cre* box (catabolite response element or CcpA binding consensus sequence) (32). The role of ScoC protection in the 5′ promoter region of the *phoPR* promoter, upstream of the P_{A6} transcription start site, was assessed by exploring the transcription in an *scoC* mutant

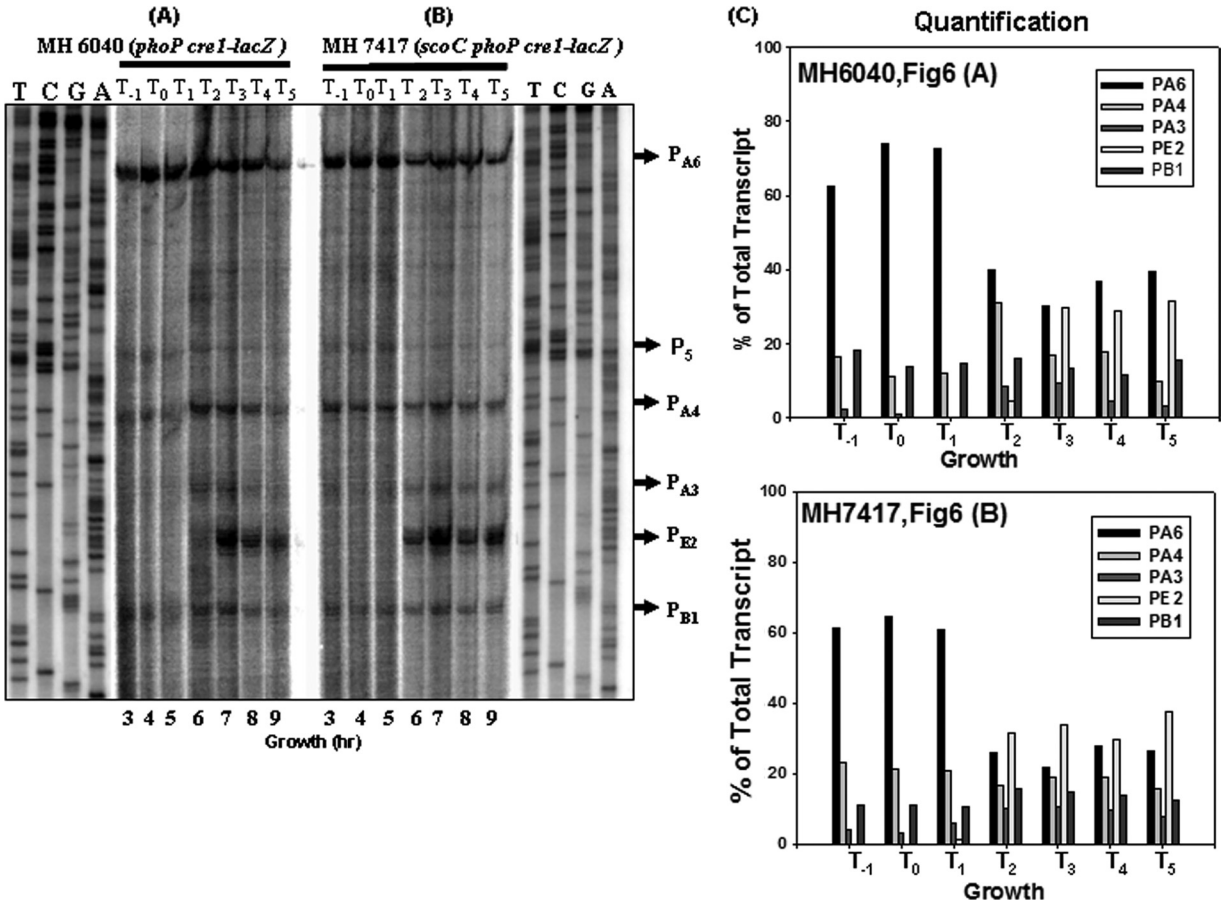

FIG. 6. Primer extension analysis of the *phoPR* promoter region from the total RNA isolated from the *phoP cre1-lacZ* (MH6040) strain or *scoC phoP cre1-lacZ* (MH7417) strain grown in LPDMG. The end-labeled primer FMH1025 was annealed to RNA isolated from exponential phase, transition stage, and postexponential phase cultures. (A) Lanes 3 to 9 show the primer extension reactions of RNA samples taken at 3 to 9 h from the *phoP cre1-lacZ* (MH6040) strain grown in LPDMG (Fig. 5) at the times indicated. T_0 is the time of Pho induction, and T_1, T_2, T_3, T_4, and T_5 are 1, 2, 3, 4, and 5 h of growth, respectively, after Pho induction in LPDMG. T_{-1} is 1 h before Pho induction (growth hour 3). Promoter expression from six transcriptional start sites, P_{B1}, P_{E2}, P_{A3}, P_{A4}, P_5, and P_{A6}, is indicated with arrows. (B) Lanes 3 to 9 show the primer extension of RNA samples taken at the indicated times from the *scoC phoP cre1-lacZ* (MH7417) strain. (C) Quantification of individual transcripts as described for Fig. 4. Bars correspond to P_{A6}, P_{A4}, P_{A3}, P_{E2}, and P_{B1}, respectively.

strain containing the *phoP cre1-lacZ* fusion (*cre1* is a mutation in the *cre* box). Expression of the *phoP cre1-lacZ* fusion in a WT strain was previously reported (32) to be similar to the that of the *phoPR-lacZ* fusion in a *ccpA* mutant strain. These expression data were consistent with the severely reduced CcpA binding to the *phoP cre1* promoter (32).

The difference in the extent of derepression from the *phoP cre1-lacZ* fusion in the wild-type strain compared to the *scoC* mutant strain suggested that the repression effect of ScoC and CcpA on the *phoPR* promoter was cumulative. Primer extension data from a *scoC* mutant or a WT strain containing the *phoP cre1* promoter fusion showed P_{A6} expression during exponential and postexponential growth in addition to the normally expressed promoters (P_{B1}, P_{E2}, P_{A3}, and P_{A4}) in the WT strain (Fig. 6). The maximum *phoP* expression from the *scoC* strain during postexponential growth (Fig. 5) was the result of the following: (i) earlier and sustained elevated expression of P_{E2}; (ii) sustained expression of P_{A4} and, to a lesser extent, P_{A3}; and (iii) sustained P_{A6} expression, albeit reduced compared to the WT strain after initiation of sporulation. No P_{A6} promoter transcription start site was detected from the unmutated *phoPR-lacZ* promoter fusion in the *scoC* mutant background during growth in LPDMG (Fig. 4), indicating that CcpA could physically block any repression relief that the *scoC* mutation provided. How ScoC affects P_{A6} expression remains a question because no derepression was observed *in vivo* in the *scoC* strain compared to the WT strain (Fig. 6), but P_{A6} was repressed by ScoC *in vitro* (data not shown). Aside from $E\sigma^A$ and CcpA, it is not clear what, or if, other regulators control P_{A6} expression. In addition to ScoC, two other proteins are known to bind to the *phoPR* promoter in the region of the P_{A6} promoter, PhoP~P (27) and AbrB (F. M. Hulett and M. A. Strauch, unpublished data). If, or how, either of these proteins affects P_{A6} transcription is not known.

Complexities of the *phoPR* promoter regulation. The major difference between the primer extension analyses of the WT *phoPR-lacZ* and the *phoP cre1* promoter fusions was the dominating presence of the P_{A6} transcript among the *phoP cre1* promoter fusion transcripts and the complete absence of P_{A6} expression of the *phoPR-lacZ* fusion transcripts. This is easily

explained based on the published role of CcpA repression of P_{A6}, the reduced affinity of CcpA binding to the promoter containing the *cre1* mutation, and the dominant presence of P_{A6} transcript in a *ccpA* mutant background (32). More puzzling were the transcript differences from the two promoters, *phoPR-lacZ* (Fig. 4) and *phoP cre1-lacZ* (Fig. 6), *in vivo*. (i) The contribution of P_{A3} to the total transcript from the *phoPR-lacZ* promoter was major compared to its weak expression from the *phoP cre1-lacZ* fusion in both the WT and the *scoC* strains; P_{A3} promoter expression from the *phoPR-lacZ* fusion was dominant in the *scoC* strain during both exponential and postexponential growth (Fig. 4B). (ii) The apparent effect on sporulation initiation in the *scoC* strain, as judged by the early and increased expression of P_{E2} compared to that in a WT strain, was observed from the *phoP cre1-lacZ* promoter but not the *phoPR-lacZ* promoter fusion.

We considered several scenarios that may have contributed to these differences. Both promoter fusions share the four densely positioned overlapping promoters (P_{B1} to P_{A4}) which may be exposed to multiple different proteins (different forms of RNAP, activators, repressors, and etc.) that compete for their overlapping binding sites, often at the same time. The RNAP machinery required for P_{A6} transcription must pass through the congested downstream region during transcription of the long untranslated region of the nascent mRNA. It appeared that P_{A3} was most negatively affected by these conditions, but the reason remains unknown. The question remains, Why does the apparent indirect ScoC regulation occur with the *phoP cre1* promoter but not the *phoP* promoter?

Further studies will be required to determine if either the dynamics of regulatory protein-DNA interactions in the congested promoter regions or the locations of the promoter fusions are responsible for the differences in levels and timing of expression observed from the individual promoters of the two promoter fusions.

In summary, the *in vivo phoPR-lacZ* transcription data, ScoC footprinting data, primer extension data, and *in vitro* transcription data reported here together suggest that ScoC is a direct repressor of *phoPR* transcription. We know that a number of proteins are involved in direct regulation of the complex *phoPR* promoter via direct binding: PhoP, PhoP~P, CcpA, AbrB, four different forms of RNAP, and now ScoC. We also have evidence that additional unknown proteins are involved: another RNAP form and possible activators and/or repressors for P_5, an unknown activator protein for P_{A3}, and a possible unknown activator for P_{A6}. There is also preliminary evidence for direct binding and activation by ResD~P of certain of the six promoters (S. Paul and F. M. Hulett, unpublished data). ScoC, Spo0A, and the ResDE TCS are also involved in indirect regulation.

The unknown regulators complicate the interpretation of *in vitro* transcription studies, making analysis P_5 and P_{A3} incomplete. Further, the interpretation of the *in vivo* analysis by primer extension data would benefit from additional information concerning the unknown regulators.

ACKNOWLEDGMENTS

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