Prediction of the *pho* regulon in *Streptomyces clavuligerus* DSM 738

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SUMMARY

Negative control of clavulanic acid by phosphate in *Streptomyces clavuligerus* DSM 738 suggests that a *pho* regulon may exist in this bacterium. *S. clavuligerus* PhoP was expressed with a C-terminal His-tag in *Escherichia coli* and purified. Binding of PhoP-His_6 to promoter fragments of *phoRP/phoU* and *pstS* was demonstrated in gel retardation experiments. These fragments contained direct 11 bp repeats resembling PHO boxes. The tentative consensus sequence, GKTCRHBBNSV, was used to search other potential PhoP target genes in the genomic sequence of *S. clavuligerus*. In total, the putative PHO binding sequence was found in promoter regions of 31 *S. clavuligerus* genes. Binding of PhoP-His_6 to the PHO box present in the promoter region of the phosphate transporter gene SSCG_07547 of *S. clavuligerus* was demonstrated. Furthermore, it was shown by real time PCR that decreased concentrations of phosphate do affect increased expressions of genes to which PhoP binds. These findings confirm that a *pho* regulon exists in *S. clavuligerus*.

KEY WORDS: Clavulanic acid, PHO box, PhoP, *Streptomyces clavuligerus*, Two-component system.

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INTRODUCTION

Inorganic phosphate negatively controls the production of the commercially important antibiotic clavulanic acid (CA) in *Streptomyces clavuligerus* (Lebrihi *et al.,* 1987) and some other secondary metabolites in the two *Streptomyces* species, *Streptomyces coelicolor* (Santos-Beneit *et al.,* 2011) and *Streptomyces natalensis* (Mendes *et al.,* 2007). In *S. coelicolor* it was shown that phosphate regulation is mediated by the two-component PhoR-PhoP system; PhoR is a sensor kinase and PhoP is a DNA-binding response regulator (Sola-Landa *et al.*, 2005; Thomas *et al.,* 2011). In *Bacillus subtilis,* phosphate limitation causes PhoP phosphorylation, and phosphorylated PhoP binds to consensus PHO boxes in promoters of *pho* regulon genes and affects the expression of these genes (Hulett, 1996). It was shown that the binding sites of PhoP within the promoters consist of two direct repeats of an 11 bp sequence that are adjacent or separated by only a few nucleotides; the binding sites came to be known as PHO boxes (Sola-Landa *et al.,* 2005). PHO boxes of three PhoP target genes in *S. coelicolor* A3 (2)

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were also detected and these were used to identify a consensus binding sequence in this bacterium (Sola-Landa *et al.,* 2005). The three genes were *phoRP*, *phoU*, and *pstS*; *phoRP* and *phoU* share a common bidirectional promoter. *PhoU* encodes a phosphate modulator and *pstS* encodes a high affinity phosphate transporter (Sola-Landa *et al.*, 2005). The consensus sequence derived was used to identify many other *S. coelicolor* PhoP target genes (Sola-Landa *et al.,* 2008). Some of these genes are involved in phosphate metabolism and others are predicted to have roles in other cellular functions such as primary metabolism and transcriptional regulation (Sola-Landa *et al.,* 2008; Martin *et al.,* 2011).

The molecular mechanisms of phosphate control in *S. clavuligerus* have not been reported. In this study, binding of purified PhoP to promoter region DNA fragments of *S. clavuligerus phoRP*, *phoU*, *pstS* and SSCG_07547 genes was shown and putative *S. clavuligerus* PHO boxes were identified. SSCG_07547 encodes a low affinity phosphate transporter. It was further shown that decreased phosphate concentrations affect increased expression of these genes. Bioinformatic analyses based on sequences of the putative PHO boxes identified PHO boxes in the promoter regions of twenty-eight additional genes in this important industrial microorganism.

MATERIALS AND METHODS

Bacterial strains and plasmid

S. clavuligerus DSM 738 was obtained from DSMZ (Leibniz Institut Deutsche Sammlung von Mikroorganismen und Zellkulturen). *Escherichia coli* Xl-1 Blue (Bullock *et al.,* 1987) and *E. coli* Rosetta™ 2(DE3) which expresses tRNAs for rarely used *E. coli* codons (Merck, Germany) were used for cloning experiments and heterologous expression, respectively. pJOE2775 (kindly provided by J Altenbuchner) which allows inducible production of His-tagged proteins was used as an expression vector.

Cloning of the *phoP* gene

phoRP genes were detected in the genomic sequence of *S. clavuligerus* (http://www.ncbi.nlm.nih.gov/nuccore/326336946, NZ_CM001015.1) by Blast analyses. The *phoP* gene was amplified from *S. clavuligerus* using primers Fphop (5'-GGGCATATGACCCGAGTGCTCGTCG-3') and Rphop (5'-AAAGGATCCGGGCTCGAACTTGTAG-3') containing NdeI and BamHI recognition sites (underlined). The PCR product was digested with NdeI and BamHI and cloned into pJOE2775 yielding pJOE-PhoP. After confirmation of the sequence of the insert, pJOE-PhoP was introduced into *E. coli* Rosetta™ 2(DE3).

PhoP expression and purification

E. coli Rosetta™ 2(DE3) cells containing pJOE-PhoP were grown in LB medium (without glucose) containing 150 µg ampicillin ml^{-1}, and induced with 1.1×10^{-2} mM rhamnose. Cells were lysed in buffer L containing 100 mM Tris/HCl, pH 7.6, 300 mM NaCl, 0.28 mM β-mercaptoethanol and a protease inhibitor cocktail (Roche, Germany) using the Emulsiflex-B15 (Avestin, Canada) apparatus. A one ml gravity flow Ni-NTA Superflow column (Qiagen, Germany) was used for PhoP purification. Bound proteins were eluted with 150 mM immidazole, and fractions containing PhoP were identified by SDS-PAGE (12% w/v) and concentrated using Amicon Ultracell-10 K centrifugal filter devices (Millipore, USA). Protein concentrations were measured using the Bradford protocol. The purified proteins were electro-transferred onto a nitrocellulose membrane and anti-6-His HRP conjugated antibody (Biomol, Germany) was used to detect PhoP-His_6. Bound antibodies were visualized using chemiluminescence reagent Western lightning plus-ECl (Biomol, Germany).

Bioinformatics analysis

A data file containing 200 bp upstream sequences of annotated *S. clavuligerus* genes was prepared using an in-house software. PHO boxes within these sequences were searched using the PatScan software (Dsouza *et al.,* 1997). From the putative PHO boxes in the promoter regions of *S. clavuligerus phoRP*, *phoU*, and *pstS* the following search pattern was derived: {(0,17,83,0), (0,0,33,67), (17,0,0,83), (0,83,0,17), (67,0,33,0), (17,67,0,17), (0,50,17,33), (0,33,33,33), (33,17,33,17), (0,50,50,0), (17,50,33,0)} 540 {(0,17,83,0), (0,0,33,67), (17,0,0,83), (0,83,0,17), (67,0,33,0), (17,67,0,17), (0,50,17,33), (0,33,33,33), (33,17,33,17), (0,50,50,0), (17,50,33,0)} 540 for promoters with one PHO box; and {(0,17,83,0),

(0,0,33,67), (17,0,0,83), (0,83,0,17), (67,0,33,0), (17,67,0,17), (0,50,17,33), (0,33,33,33), (33,17,33,17), (0,50,50,0), (17,50,33,0)} 380 {(0,17,83,0), (0,0,33,67), (17,0,0,83), (0,83,0,17), (67,0,33,0), (17,67,0,17), (0,50,17,33), (0,33,33,33), (33,17,33,17), (0,50,50,0), (17,50,33,0)} 530 {(0,17,83,0), (0,0,33,67), (17,0,0,83), (0,83,0,17), (67,0,33,0), (17,67,0,17), (0,50,17,33), (0,33,33,33), (33,17,33,17), (0,50,50,0), (17,50,33,0)} 380 {(0,17,83,0), (0,0,33,67), (17,0,0,83), (0,83,0,17), (67,0,33,0), (17,67,0,17), (0,50,17,33), (0,33,33,33), (33,17,33,17), (0,50,50,0), (17,50,33,0)} 530 for promoters with two PHO boxes. In the parenthesis, positions 1, 2, 3 and 4 are, respectively, for nucleotides A, C, G, and T. The search pattern for one PHO box identifies two direct repeat units in one PHO box, and the search pattern for two PHO boxes identifies four direct repeats within two PHO boxes. The threshold score of 540 was set for two repeats within one PHO box. For instances of four repeats with two PHO boxes, the threshold score of 380 for repeat units 1 and 3 and the score of 530 for repeat units 2 and 4 were assigned.

Electrophoretic mobility shift assay (EMSA)

DNA fragments of genes containing promoter regions with putative PHO boxes and a negative control DNA were amplified by PCR and tested for PhoP binding by electrophoretic mobility shift assays (EMSA). The sequences of primers used for amplification of the promoter regions are listed in supplementary Table S1. The negative control DNA was an amplified fragment from within the coding region of the *phoP* encoding region. This fragment was amplified from *S. clavuligerus* using primers FNeg (5'- GAGATGTTGCTCCGCAAC -3') and RNeg (5'- ACTTAGGGCTCGAACTTG -3'). Purified PhoP protein (0.9 to 4.3 pmol) was incubated with the DNA fragments (10 ng) in the presence of 10 mM Tris/HCl, pH 8.0, 0.4 mM $MgCl_2$, 10 mM KCl, 1 mM DTT, 0.01% (v/v) Nonidet P40 and 13% (v/v) glycerol. Total reaction volume was 10 µl and incubation was for 25 min at room temperature. Electrophoresis was done on 1% (w/v) agarose or 6% (w/v) polyacrylamide gels.

Expression of *phoRP*, *phoU*, *pstS* and SSCG_07547 genes in *S. clavuligerus* under variant phosphate concentrations

Flasks containing 250 ml of seeding medium (peptone 10 g l^-, glycerol 20 g l^-, malt 10 g l^-, pH 7.0; Cole, 1977) were each inoculated with one ml of *S. clavuligerus* spore suspension (ca. 10^7-10^8 spores). The cultures were incubated at 28°C with shaking at 220 rpm for 20-22 h. The biomass was harvested, washed with sterile deionized water, weighed, and resuspended in 100 ml of Starch-Asparagine defined medium (Paradkar & Jensen, 1995) containing low (3mM) or high (25mM) concentrations of KH_2PO_4. Incubation was continued under the same conditions, and samples for measurement of growth and phosphate concentration and for gene expression analysis were taken 40, 60, and 100 hours after start of incubation.

To measure mycelia biomass, bacteria in 2 ml culture medium were precipitated, washed with NaCl (0.1%), and dried for 2 days at 60°C. Phosphate concentrations were determined in culture supernatant by the modified malachite green/molybdate method (Lanzetta *et al.*, 1979) in which Sterox was replaced by Tween 20 (0·01%). Expression of *phoRP*, *phoU*, *pstS* and SSCG_07547 genes was assessed by real time PCR. RNA was isolated using RNeasy Kit (Qiagen, Germany), and cDNA was synthesized using a cDNA Synthesis Kit (Fermentas, Germany). Sequences of primers used for real time PCR amplification of *phoR, phoU, pstS* and SSCG_07547 mRNAs and 16srRNA are provided in supplementary Table S1. 16srRNA genes were used as reference genes. Real time PCR was performed on a Corbett 65H0 machine (Corbett Research, Sidney, Australia) using the QuantiFast SYBR Green PCR Kit (Qiagen, Germany). The amplification program consisted of one cycle of 95°C for 10 minutes and 40 cycles of 95°C for 15 seconds, 60°C for 25 seconds and 72°C for 25 seconds. Fold change in expression of the genes were obtained using software provided by the Corbett instrument. Three PCR reactions were done for each gene.

RESULTS

Detection of key components of the *Pho* regulon of *S. clavuligerus*

Initially, PhoRP, PhoU, and PstS encoding genes of *S. clavuligerus* were identified by a Blast-P search (Altschul *et al.*, 1997) using the homologues of *S. coelicolor* A3(2). *S. clavuligerus* PhoR, PhoP, PhoU and PstS have 82%, 97%, 85% and

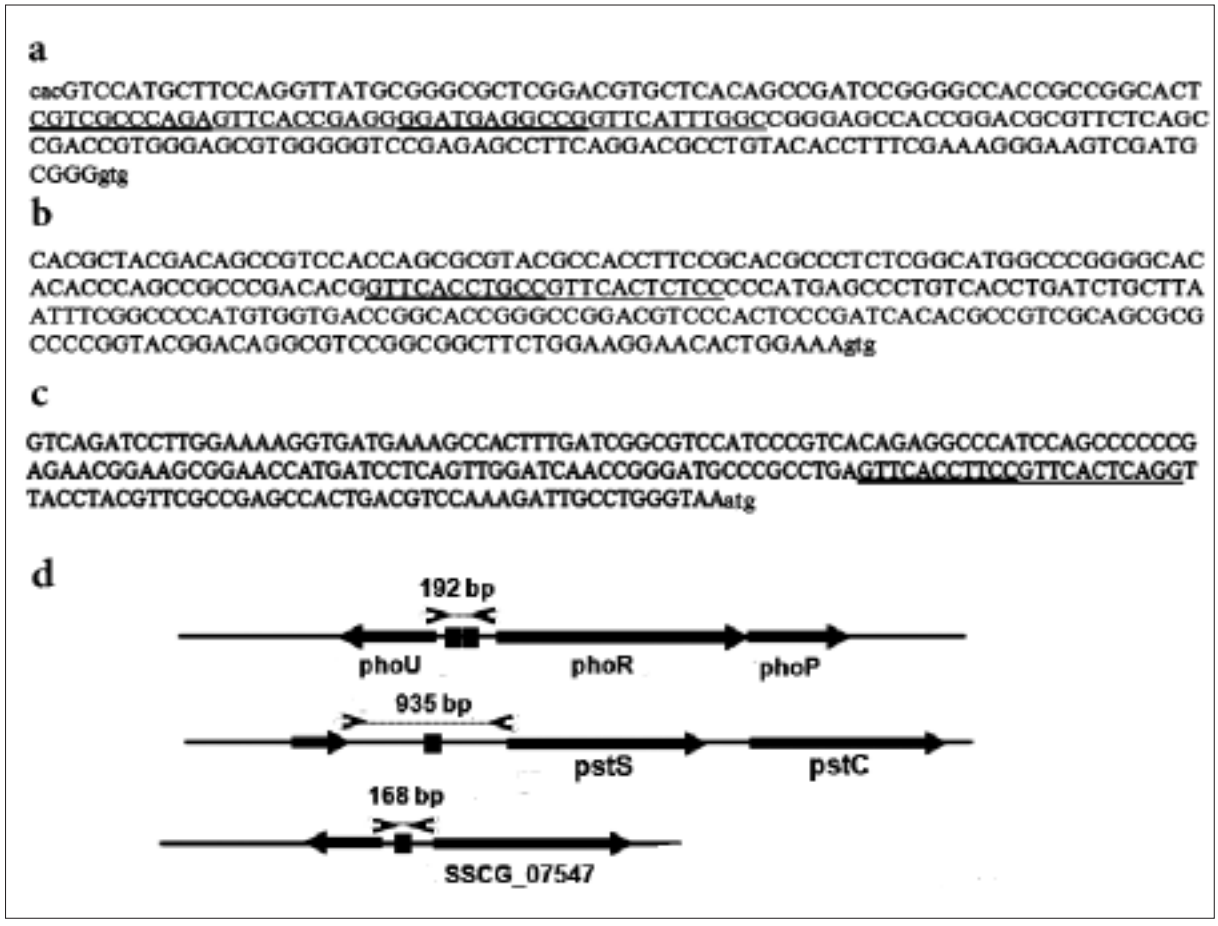

FIGURE 1 - *Predicted PHO boxes in promoter regions of putative target genes. a. Predicted PHO boxes in the promoter region of phoRP-phoU. b. Predicted PHO boxes in the promoter region of pstS. c. Predicted PHO boxes in promoter region of SSCG_07547. Sequences encoding the initiating methionine of the proteins are shown with non-capitalized letters. The direct repeat units of the PHO boxes are underlined; the first unit in each box is underlined in bold. phoRP and phoU genes are transcribed from different DNA strands. d. Schematic drawing of the promoter fragments used in EMSA. Small arrows indicate primers used for the amplification of the promoter regions. Black boxes mark PHO boxes.*

66% sequence identity with respective proteins of *S. coelicolor*. The encoding genes have the same organization in *S. clavuligerus* as reported for *S. coelicolor* (http://www.broadinstitute.org/anotation/genome/streptomyces_group/GeneDetails.html?sp=S7000003820108528, http://www.broadinstitute.org/anotation/genome/streptomyces_group/GeneDetails.html?sp=S7000003820121042, Sola-Landa *et al.,* 2005).

Two putative PHO boxes resembling the *S. coelicolor* consensus sequence were found within the promoter region of *phoRP/phoU* and one within the promoter region of *pstS* in *S. clavuligerus* (Figure 1 a, b, d).

Purification of histidine-tagged *S. clavuligerus* PhoP

S. clavuligerus phoP was cloned into the expression vector pJOE2775 and introduced into *E. coli* Rosetta™ 2(DE3) (Materials & Methods). After induction with rhamnose, PhoP-His_6 was detected as a strong protein band (MW~26 KDa) in the electrophoretic profile of crude lysates (Figure 2a).

Following purification by Ni-NTA chromatography, about 1 mg PhoP was obtained from a 1 L culture (Figure 2b). The identity of the purified PhoP-His_6 was confirmed by immunoblotting using an anti-His antibody (Figure 2c).

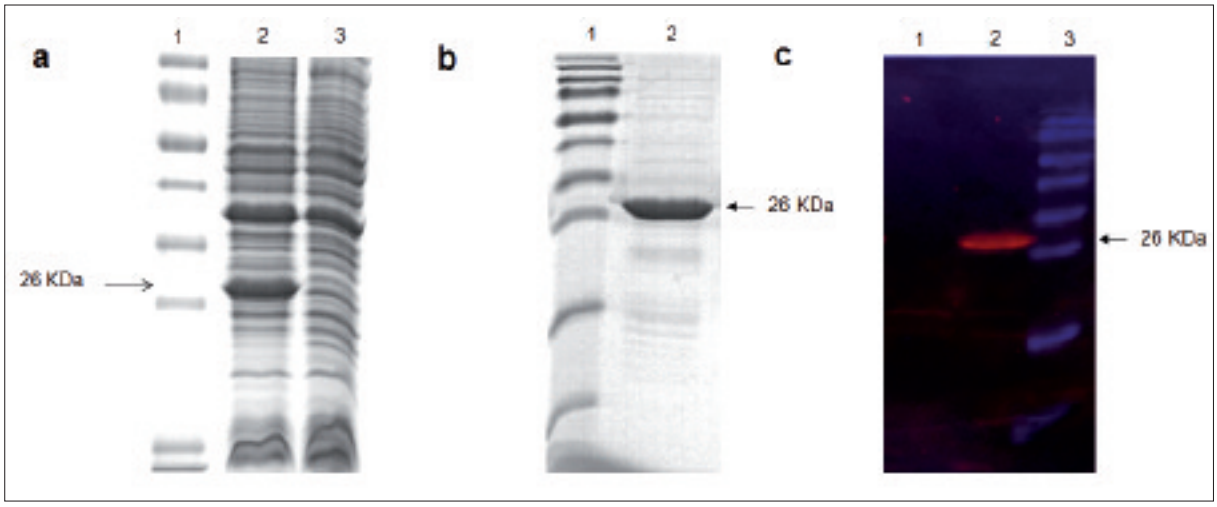

FIGURE 2 -*SDS-PAGE and immunoblot identification of PhoP-His$_6$ expressed in E. coli. E. coli Rosetta cells were transformed with pJOE-PhoP. a. lane 1: protein size marker; lane 2: extracted proteins from cells induced with rhamnose; lane 3: extracted proteins from uninduced cells. b. lane 1: protein size marker; lane 2: PhoP-His$_6$ purified by Ni-NTA affinity chromatography. c. Immunoblot with anti-His antibodies. lane 1: extracted proteins from uninduced cells; lane 2: PhoP-His$_6$ purified by Ni-NTA affinity chromatography; lane 3: protein size marker. Molecular weights of PhoP are provided next to arrows.*

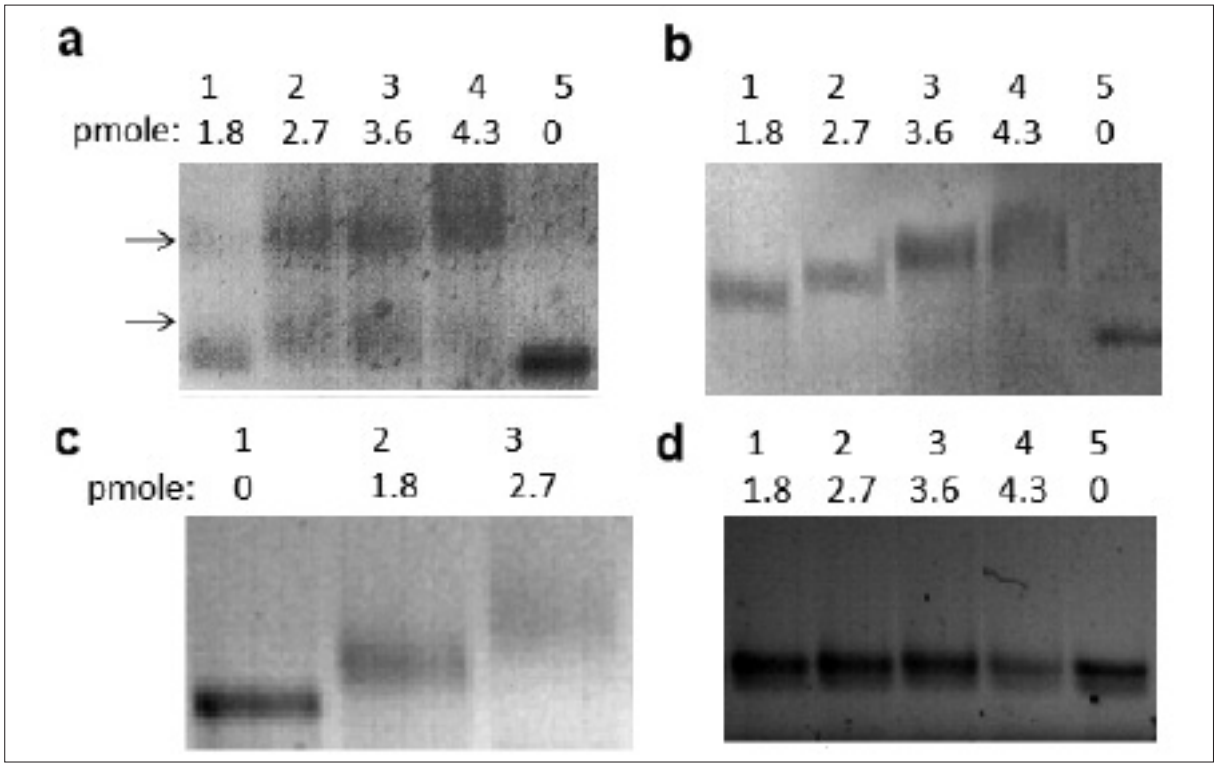

FIGURE 3 - *Electrophoretic mobility shift assay. Promoter region fragments of phoRP/phoU (a), pstS (b), SSCG_07547 (c), and negative control DNA (d) were incubated with increasing amounts of PhoP-His$_6$ (lanes 1-4). A single shifted band was observed upon incubation of pstS and SSCG_07547 fragments at each protein concentration, whereas two bands (arrows) were observed upon incubation of the phoRP/phoU fragment. No shifted band was observed upon exposure of the negative control DNA fragment to different concentrations of PhoP.*

PhoP binds to intergenic *phoRP-phoU* and *pstS* promoter regions

To address whether *S. clavuligerus* PhoP binds to the promoter regions of *phoRP/phoU* and *pstS*, EMSA assays were performed with PCR fragments containing the upstream regions of *phoRP/phoU* and *pstS*, respectively. Two shifted bands were observed upon exposure of the *phoRP/phoU* fragment to PhoP (Figure 3a). In contrast, PhoP binding to the *pstS* fragment produced a single retarded band (Figure 3b) and no shifted band was observed upon exposure of the negative control DNA fragment to PhoP (Figure 3d). The same band shift pattern for *phoRP/phoU* and *pstS* fragments was previously reported in *S. coelicolor* (Sola-Landa *et al.,* 2005).

Identification of putative PhoP target genes in the *S. clavuligerus* genome

The six direct repeat units in the putative PHO boxes of *phoRP/phoU* and *pstS* were aligned and the consensus sequence GKTCRHBBNSV was defined as a very tentative PHO box in *S. clavuligerus* (Figure 4). Nucleotides that appeared in two or more of the six repeat units were included in the tentative consensus sequence. To identify the *pho* regulon of *S. clavuligerus*, upstream regions of *S. clavuligerus* genes were searched by PatScan for the presence of PHO boxes consisting of two repeats of GKTCRHBBNSV. These analyses resulted in 31 hits (Table 1). As expected, *phoRP*, *phoU* and *pstS* were included among the genes. Some of the genes identified by PatScan encode proteins that have phosphate related functions, such as a phosphatase (SSCG_02052), a sensor kinase (SSCG_01097), a regulator of phosphate transport (SSCG_01098), and phosphate transporters (SSCG_01155 and SSCG_07547). Genes involved in cell differentiation such as SSCG_06986 that encodes a WhiB protein, and in transcriptional regulation such as SSCG_04454 and SSCG_06876 that encode sigma factors and SSCG_05962 that encodes a GntR- family transcriptional regulator, were also among genes that may constitute the *pho* regulon of *S. clavuligerus*. Several genes that encode enzymes, including lactoylglutathionelyase (SSCG_05898), methylase (SSCG_01584), acetoacetyl-CoA synthetase (SSCG_06339), glutamyl endopeptidase II (SSCG_06674), acetyltransferase (SSCG_06813), monooxygenase (SSCG_07201), ATPase (SSCG_07298), phospholipase C (SSCG_07881), oxidoreductase (SSCG_05610) and amino transferase (SSCG_06777), were also identified.

PhoP binding assay for selected target genes

Thirteen promoter regions identified by PatScan were selected for EMSA analysis based on their degree of similarity to the consensus sequence (Table 1). PhoP binding was confirmed only to the upstream region of SSCG_07547 (Figure 3c). This gene encodes a low affinity phosphate trans-

a

	1	2	3	4	5	6	7	8	9	10	11
pstS	G	T	T	C	A	C	C	T	G	C	C
pstS	G	T	T	C	A	C	T	C	T	C	C
phoRP-U	C	G	T	C	G	C	C	C	A	G	A
phoRP-U	G	T	T	C	A	C	C	G	A	G	G
phoRP-U	G	G	A	T	G	A	G	G	C	C	G
phoRP-U	G	T	T	C	A	T	T	T	G	G	C
Consensus seq.	G	K	T	C	R	H	B	B	N	S	V

b

	Nucleotide positions in direct repeat units										
Nucleotide	1	2	3	4	5	6	7	8	9	10	11
A	0	0	1	0	4	1	0	0	2	0	1
C	1	0	0	5	0	4	3	2	1	3	3
G	5	2	0	0	2	0	1	2	2	3	2
T	0	4	5	1	0	1	2	2	1	0	0

FIGURE 4 - *Consensus sequence of the direct repeat unit of S. clavuligerus PHO boxes. a. The six direct repeat units present in the three PHO boxes in promoter regions of phoRP/phoU and pstS in S. clavuligerus are aligned and the 11 nucleotide consensus sequence is shown in the last line. b. The number of times each nucleotide is present at each of the 11 positions in the six direct repeat units is shown.*

TABLE 1 - *Streptomyces clavuligerus annotated genes*[a] *predicted to contain PHO boxes by PatScan.*

Accession no.	*Gene annotation*	*No. of predicted PHO boxes*
SSCG_01155[b]	phosphate transporter protein PstS	1
SSCG_01343	Hypothetical protein	1
SSCG_01584[b]	Putative methylase	1
SSCG_01644	FabD protein	1
SSCG_02052[b]	Inositol monophosphatase-like protein	1
SSCG_03403	Beta-lactamase inhibitory protein	1
SSCG_03734	Putative integral membrane protein	1
SSCG_04454	RNA polymerase ECF-subfamily sigma factor	1
SSCG_05898[b]	Putative lactoylglutathionelyase	1
SSCG_06339	Acetoacetyl-CoA synthetase	1
SSCG_06674[b]	Glutamyl endopeptidase II	1
SSCG_06813[b]	Putative acetyltransferase	1
SSCG_06876	ECF subfamily RNA polymerase sigma factor	1
SSCG_06986[b]	Putative WhiB protein	1
SSCG_07201[b]	Putative monooxygenase	1
SSCG_07298[b]	ATP-binding region, ATPase-like protein	1
SSCG_07409	Conserved Hypothetical protein	1
SSCG_07547[b]	Putative low-affinity phosphate transporter	1
SSCG_07830	Hemolysin-type calcium-binding region	1
SSCG_07881	Putative non-hemolytic phospholipase C	1
SSCG_00211	pentapeptide repeat-containing protein	2
SSCG_00996	Hypothetical protein	2
SSCG_01097[b]	two-component system sensor kinase PhoR	2
SSCG_01098[b]	phosphate transport regulatory protein PhoU	2
SSCG_02426	Putative membrane protein	2
SSCG_04369[b]	ABC transporter substrate-binding protein	2
SSCG_05610	Putative flavoproteinoxidoreductase	2
SSCG_05962[b]	Putative GntR-family transcriptional regulator	2
SSCG_06777	Amino transferase	2
SSCG_07106[b]	integral membrane protein	2
SSCG_07822[b]	Hypothetical protein	2

Obtained from The Broad Institute, MIT (http://www.broadinstitute.org/annotation/genome/streptomyces_group/FeatureSearch.html).
Genes tested for binding by PhoP in EMSA assay.

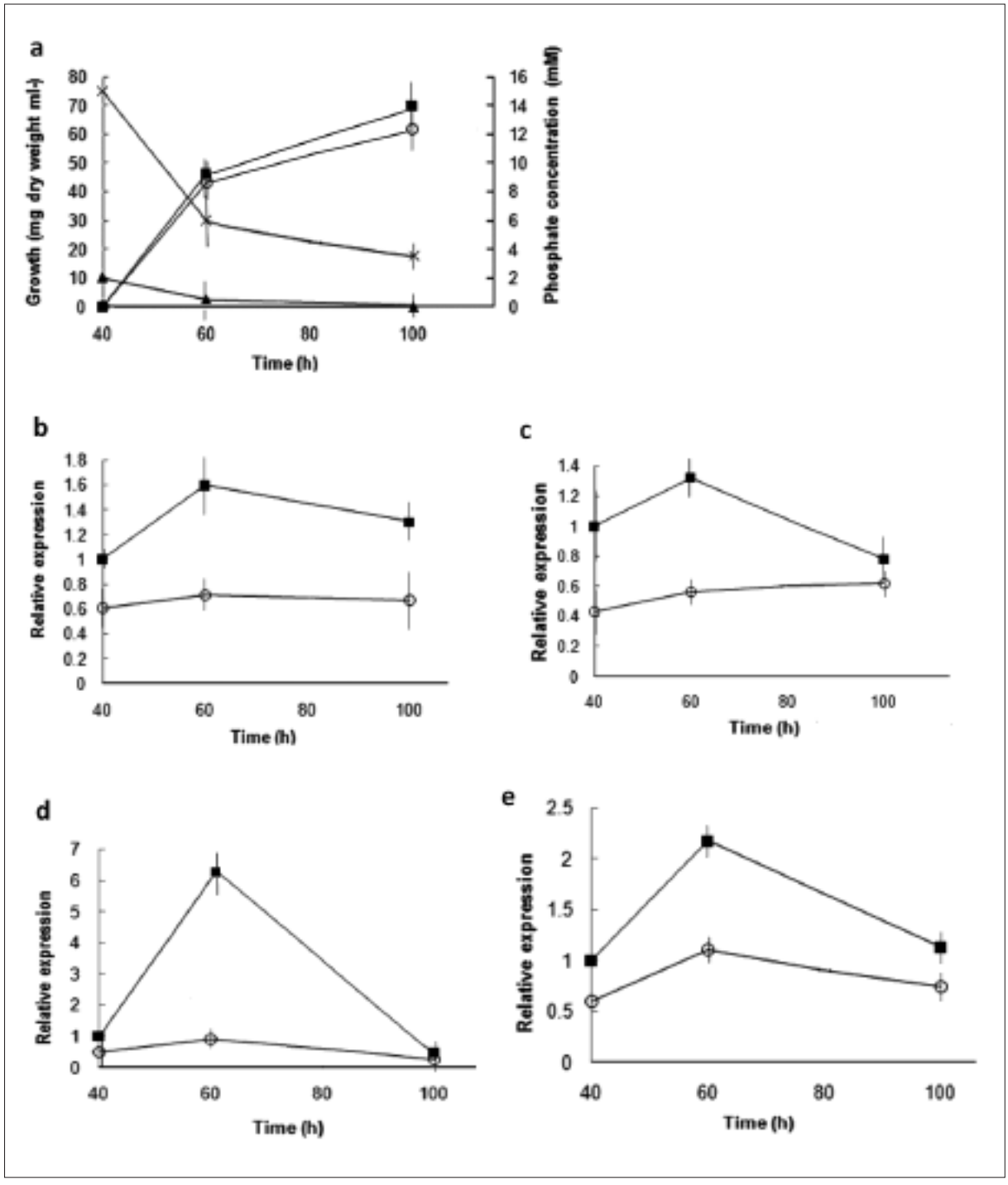

FIGURE 5 - *Measurement of biomass and phosphate concentration, and gene expression analysis during growth with low or high concentrations of inorganic phosphate. a. Biomass at indicated time points after start of incubation in presence of low (3 mM; ○) and high (25 mM; ■) concentrations of KH_2PO_4, and residual phosphate concentrations at indicated time points after growth in media with initially low (▲) and high (×) concentrations of KH_2PO_4. Expression of phoR (b), phoU (c), pstS (d), and SSCG_07547 (e) in media with low (■) and high (○) concentrations of KH_2PO_4. Gene expression was assessed by real time PCR. The experiments were done in triplicate, and vertical bars indicate standard deviation.*

fer protein. The 168 bp fragment contained a single PHO box (Figure 1c, d). A possible explanation for not observing binding to most of the candidate promoters is presented in the Discussion section.

Empirical confirmation of effect of inorganic phosphate on expression of *phoR*, *phoU*, *pstS*, and SSCG_07547 genes

Growth curves of *S. clavuligerus* and phosphate depletion during growth are shown in Figure 5a. Levels of expression of *phoR*, *phoU*, *pstS*, and SSCG_07547 genes were assessed by real time PCR in cells grown in media with initial phosphate concentrations of 3mM and 25mM. Although levels of expression of the *phoR*, *phoU*, *pstS*, and SSCG_07547 genes were somewhat lower in media with the 25 mM initial phosphate concentration at all time points tested, most notable difference in expression of all four genes was observed after 60 minutes of growth (Figure 5, b-e). This time point is just prior to start of stationary growth phase. The correlation of the expression of these genes with phosphate levels is evident within the culture with the limiting (3 mM) initial phosphate concentration. Peak expression of the genes occurs when phosphate concentration decreased to approximately 0.5 mM.

DISCUSSION

The existence of a *pho* regulon in *S. clavuligerus* has not previously been demonstrated. However, the existence of this regulon in the evolutionarily related bacterium *S. coelicolor* and the fact that inorganic phosphate affects the production of clavulanic acid in *S. clavuligerus* suggested that this bacterium is likely to contain such a regulon. Presuming similarities with *S. coelicolor*, we expected *PhoRP*, *PhoU*, and *PstS* to be components of the *pho* regulon of *S. clavuligerus*. Putative PHO boxes resembling the *S. coelicolor* consensus PHO box were indeed found in the promoter regions of these genes in *S. clavuligerus*. As with *S. coelicolor*, two PHO boxes were observed in the bidirectional promoter region of *phoRP/phoU* and one was found in the promoter region of *pstS* in *S. clavuligerus* (Sola-Landa *et al.,* 2005). EMSA assays showed that purified PhoP protein binds upstream sequences of these genes. The two shifted bands observed for the *phoRP/phoU* fragment probably reflect PhoP binding to either one or both PHO boxes within the promoter region. A consensus sequence based on the repeat units of these genes was used to identify with bioinformatics tools other putative genes of the *S. clavuligerus pho* regulon. Although based on only six repeat units and considerable variability within the sequences, the sequence was considered potentially useful as the *S. coelicolor* consensus sequence based on exactly the same units in homologous genes had previously proved to be valuable for identification of PhoP regulated genes (Sola-Landa *et al.,* 2008). Thirty-one genes were identified by the bioinformatics approach, but PhoP binding to the promoter region was evidenced for only one (SSCG_07547) of 13 genes tested. In a previous *S. coelicolor* study, PhoP-mediated mobility shift was observed for eight of twenty candidate promoter regions tested (Sola-Landa *et al.*, 2008). Whereas Sola-Landa *et al.* (2008), used the isolated DNA binding domain of PhoP, we analyzed the binding of a full length PhoP-His_6 fusion protein. It has been reported that phosphorylated PhoP and the isolated DNA binding domain of PhoP, each binds PHO boxes more efficiently than non-phosphorylated whole length protein (Himpens *et al.,* 2000; Sola-Landa *et al.*, 2008). Since PhoP-His_6 was isolated from *E. coli* in the absence of the corresponding *phoR* gene, it is possible that it was not phosphorylated. Promoter regions of *phoRP/phoU*, *pstS* and SSCG_07547 might represent the most important target sites, which are even bound by non-phosphorylated full-length PhoP. The differential binding of PhoP to PHO boxes of different genes probably reflects the variable effects of phosphate on expression of these genes. Although all components of the *pho* regulon in *S. clavuligerus* have most likely not been identified in this study, the proposition that *phoR*, *phoU*, *pstS*, and SSCG_07547 genes are components of this *pho* regulon was given added support by showing that phosphate concentrations affect the level of expression of these genes. Similar to results presented here, induction of *phoRP*, *phoU* (Sola-Landa *et al.,* 2005), *pitH2* and *pstS* (Santos-Beneit *et al.,* 2008) expression upon phosphate starvation was previously shown in *S. coelicolor*. In that organism, phosphate deprivation causes phosphorylation of the transcription regulator

PhoP, and phosphorylated PhoP positively controls expression of target genes by binding to PHO boxes present in their promoters (Santos-Beneit *et al.,* 2008; Santos-Beneit *et al.,* 2011; Sola-Landa *et al.,* 2005).

This study was designed partly with the consideration that the findings may reveal novel strategies for enhanced production of clavulanic acid by *S. clavuligerus*. CA, a very potent β-lactamase inhibitor, is the most important product among 21 secondary metabolites produced by *S. clavuligerus*. In fact, CA is combined with amoxicillin to produce one of the most highly used antibiotics, co-amoxyclav (Saudagar *et al.,* 2008). Media optimization can significantly affect the type and concentration of the various secondary metabolites produced by industrial organisms, and optimization protocols have been applied to enhance CA production (Saudagar and Singhal, 2007). The mechanisms by which certain medium ingredients negatively (Bushell *et al.,* 2006) or positively (Hamedi *et al.,* 2011) affect CA production in *S. clavuligerus* are incompletely understood. Although clavulanic acid production is negatively regulated by phosphate, our bioinformatics search did not find PHO boxes within the clavulanic acid biosynthesis gene cluster nor in the promoter region of the pathway specific regulatory genes *ccaR* and *claR*. This suggests that CA production is not directly controlled by PhoP. Interestingly, SSCG_05962, encoding a transcriptional regulator (GntR-family) and SSCG_04454 and SSCG_06876, encoding ECF-subfamily RNA polymerase sigma factors were included among the genes predicted to bind PhoP. Whether these components of the cellular transcription apparatus play a role in the control of clavulanic acid production waits to be elucidated. Further studies need to be performed to determine how phosphate regulates CA production in *S. clavuligerus*.

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