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REVIEW ARTICLE

The master regulator PhoP coordinates phosphate and nitrogen metabolism, respiration, cell differentiation and antibiotic biosynthesis: comparison in *Streptomyces coelicolor* and *Streptomyces avermitilis*

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Phosphate limitation is important for production of antibiotics and other secondary metabolites in *Streptomyces.* Phosphate control is mediated by the two-component system PhoR-PhoP. Following phosphate depletion, PhoP stimulates expression of genes involved in scavenging, transport and mobilization of phosphate, and represses the utilization of nitrogen sources. PhoP reduces expression of genes for aerobic respiration and activates nitrate respiration genes. PhoP activates genes for teichuronic acid formation and reduces expression of genes for phosphate-rich teichoic acid biosynthesis. In *Streptomyces coelicolor,* PhoP repressed several differentiation and pleiotropic regulatory genes, which affects development and indirectly antibiotic biosynthesis. A new bioinformatics analysis of the putative PhoP-binding sequences in *Streptomyces avermitilis* was made. Many sequences in *S. avermitilis* genome showed high weight values and were classified according to the available genetic information. These genes encode phosphate scavenging proteins, phosphate transporters and nitrogen metabolism genes. Among of the genes highlighted in the new studies was *aveR*, located in the avermectin gene cluster, encoding a LAL-type regulator, and *afsS,* which is regulated by PhoP and AfsR. The sequence logo for *S. avermitilis* PHO boxes is similar to that of *S. coelicolor,* with differences in the weight value for specific nucleotides in the sequence.
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INTRODUCTION

Phosphate is one of the essential nutrients of all living beings. In early studies, Martín and Demain[1] reported that, following phosphate starvation, there is a change in the metabolism of the antibiotic producing strains, which causes the slowdown of primary metabolism and triggers production of secondary metabolites. This early hypothesis has been largely confirmed by the research developed in the last few decades and is discussed in more detail below taking into account recent developments in the coordination of secondary metabolism. The aim of this article is to present an up-to-date integrative view of the PhoP regulation of metabolism in *Streptomyces*.

Genetic basis of phosphate control of metabolism

In 2003, Sola-Landa *et al.*[2] reported that the two-component system PhoR-PhoP controls not only primary metabolism but also the biosynthesis of the pigmented antibiotics actinorhodin and undecylprodigiosin in *Streptomyces lividans.*[2,3] The two-component PhoR-PhoP study was later extended to *S. coelicolor,*[4,5] *Streptomyces natalensis,*[6] *Streptomyces clavuligerus*[7] and some other *Streptomyces* species, including recently *S. avermitilis.*[8]

The *Streptomyces* PhoR-PhoP system belongs to classIIIA of two-component systems.[9–11] PhoR is a membrane sensor kinase of 426 amino acids (45.3 kDa), with a single transmembrane domain and a stretch of hydrophobic amino acids for membrane interaction. PhoP is a 223 amino acids protein (24.7 kDa), member of the OmpR family of DNA-binding response regulators.[12]

Under Pi starvation conditions, the PhoR sensor kinase self-phosphorylates, and then transfers its phosphate group to PhoP.[13] In all the text, we refer to the phosphorylated form of PhoP (PhoP-P) as regulator, but for the sake of simplicity we will use the term PhoP. The phosphorylated PhoP binds specific sequences named PHO boxes and activates or, in some cases, represses the expression of genes under direct control of PhoP, which forms the *pho* regulon.[14]

The *Streptomyces* PHO boxes are formed by direct repeat units (DRus) of 11 nucleotides, seven nucleotides being well conserved; other characteristics of the PHO boxes have been described previously.[15,16] In *S. coelicolor*, the most conserved sequence is GTTCACC, obtained from the alignment of the PhoP-binding sites of the best studied PhoP-regulated genes.[5,16,17] The sequence logo representing the PhoP-binding DRus is shown in Figure 1. Two DRus are required for binding of PhoP to DNA; one DRu is not sufficient.[5] Using theory-based model,[18] it was possible to calculate a Ri value, (the information content) for each PHO box. The Ri values reflect the sequence conservation; key nucleotides for binding tend to be more conserved.

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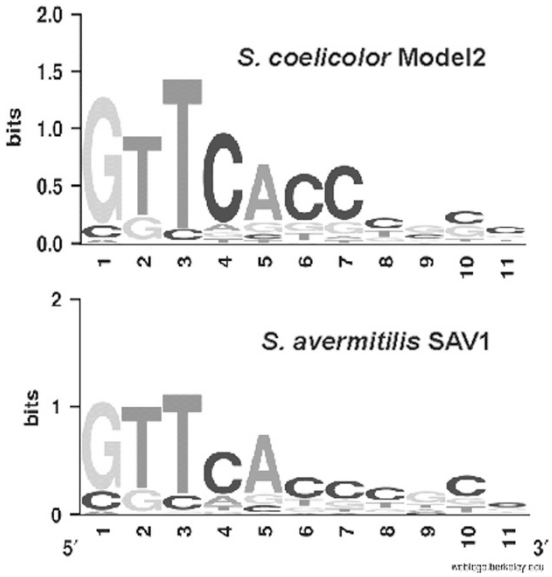

Figure 1 Comparative logos of a DRu in the Pho boxes of *S. coelicolor* (upper panel) and *S. avermitilis* (lower panel). The frequency of a nucleotide in each position is represented by the height of the letter at that position. A full color version of this figure is avaiable at *Journal of Antibiotics* online.

On the basis of their Ri values and their arrangement, the DRus in the binding sites were classified in several types: core DRus are those well conserved, showing positive Ri values and non-core DRus (so-called extension units) deviate to some degree from the consensus sequences. Allenby *et al.*[19] reported the presence of a 9 nucleotides motif in 150 out of 333 PhoP-ChIP enriched sequences. This motif appear to correspond to core DRus.

Initially, PhoP was found to be a positive regulator that was involved in activation of phosphate metabolism genes (see below), but more recent evidence indicates that is also able to repress some genes involved in primary metabolism and in the biosynthesis of secondary metabolites. In fact, when PhoP binds to the −35 region of the promoters of PhoP-regulated genes, it acts as an activator by recruiting RNA polymerase to the promoter regions, whereas when it binds to the −10 regions, or inside the open reading frame, usually it works as a repressor by preventing transcription.[19–21]

THE *pho* REGULON: PhoP-REGULATED GENES

Transcriptomic studies, footprinting assays and *in vivo* expression studies with reporter genes allowed the initial identification of about 50 genes, which are regulated by PhoP and form the core of the *pho* regulon.[17] A list of relevant genes regulated by PhoP is shown in Table 1 and a summary of downregulated and upregulated genes is shown in Figure 2. New approaches using chromatin immunoprecipitation (ChIP-on-chip) have enlarged the scope of these studies.[19]

Phosphate scavenging, transport, storage and mobilization

Rodríguez-García *et al.*,[4] in phosphate shift-down experiments using transcriptomic and proteomic analysis, discovered that the primary response to *phoP* deletion in *S. coelicolor* was the increase in expression of many genes that are involved in phosphate scavenging, transport, storage and mobilization. The primary targets of PhoP regulation were genes encoding phosphatases, including *phoA*, *phoC,* the phospholipase *phoD*[15] and the phytase-encoding gene, *phyA*.[22] The *S. coelicolor* and *S. lividans phyA* expression is regulated by phosphate, its transcription is PhoP-dependent and there is a PHO box in the promoter region of the *phyA* gene.[4,22]

Two major phosphate transport systems are present in *Streptomyces* species, the high-affinity *pstSCAB* (phosphate specific transport) and the low-affinity *pit* (inorganic phosphate transport). The main phosphate transport system, *pstSCAB,* is a clear target for PhoP. Indeed, *pstS* is the most highly induced gene in *S. coelicolor* following inorganic phosphate depletion, up to 240-fold expression increase. PHO boxes have been identified in the promoter region of the *pstSCAB* cluster[5] and an internal PHO box has been proposed.[19] In addition, one of the *pit* genes, *pitH2,* which encodes a low-affinity phosphate transport system, is also upregulated by PhoP.[16]

Another enzyme involved in phosphate storage and mobilization that is upregulated by PhoP after phosphate depletion is the polyphosphate kinase encoded by the *ppk* gene.[23] This protein has three enzyme activities: polyphosphate synthase, polyphosphate hydrolase and phospholipase.[24,25]

Oxidative phosphorylation and nitrate respiration genes

Another important class of genes regulated by inorganic phosphate, whose expression is mediated by PhoP, are the oxidative phosphorylation genes, including several cytocrome C and B oxidase complexes, NADH dehydrogenase complex, succinate dehydrogenase and genes for nitrate respiration (Table 1). Several of these genes are also regulated by the redox response regulator Rex.[26] Most of the oxidative phosphorylation genes have a profile that indicates that they are repressed by PhoP following phosphate depletion, whereas the nitrate reductase genes are activated.[4,19] These results suggest that depletion of phosphate results in a switch from aerobic respiration to nitrate respiration.

Interestingly, a set of genes that forms the F_oF_1-ATP synthase cluster (*atpBFHAGD*) shows the same transcription decrease, both in the wild-type strain and the Δ*phoP* mutant, following phosphate starvation. This indicates that the ATP syntase components are not regulated directly by PhoP, although they respond to phosphate limitation. However, ChIP-on-chip experiments have shown that PhoP binds to the ATP synthase promoter.[19]

Genes involved in protein synthesis and RNA polymerase subunits formation

Most of the 62 genes in this class, largely integrated in 11 clusters,[4] decrease clearly their expression following phosphate starvation, indicating that primary metabolism is switched off upon inorganic phosphate depletion. Most of the genes involved in protein synthesis and amino acid metabolism are not regulated directly by PhoP, but some others, respond to PhoP by activating their expression. Important amino acid related genes, downregulated by PhoP, are the glutamine synthetase genes (see below).

Genes for teichuronic and teichoic acid synthesis

Cell wall teichuronic acids are phosphate free, but teoichoic acids are phosphate rich and, when the cell is deprived of inorganic phosphate, teichoic acids may serve as phosphate reserve material. A set of genes, tentatively encoding enzymes for teichuronic acid biosynthesis,[27] are activated by PhoP following phosphate starvation and contain PhoP-binding sites in their promoter regions, and some genes, presumably involved in teichoic acid biosynthesis, are repressed by phosphate depletion.[4,19]

Genes for iron metabolism and oxidative stress

In several bacteria, it is well known that phosphate limitation triggers oxidative stress. This is likely due to changes in the iron metabolism, since excess of iron in the cell causes the Fenton effect that results in

Table 1 Genes up-regulated and down-regulated by PhoP under phosphate limiting conditions in *S. coelicolor*

Function	*Gene Down-regulated in the wild type*	*Gene Up-regulated in the wild type*
Amino acid biosynthesis	*hisC2, aspC,* SCO0992, SCO3792	*gluABCD, ask, sahH, trpE*
Protein synthesis: Ribosomal proteins, Translational factors, aa-tRNA synthesis, protein maturation, rRNA modification,Transcription	TU-1, *ts, fusA* *aspS* *serS*	*rpmF* *rplBCDMNOPQRSTVWX, rpsCJEHKMNQS rpmDCl* *fusA, rpoA, rpoB, rpoC, ssb, moA*
Nucleotides metabolism	*pyrR, purEFM*	*purQ purH purL pyrH*
Respiration	*qcrA, qcrC, cox1, cydB,* SCO3965 to SCO3968 SCO4562-SCO4575 (NADH DH), SCO2148, SCO2151 and SCO2155-SCO2166 (Cytochrome C and B complexes), SCO4855-SCO4858 (Succinate DH) SCO5366-5374 (F-type ATPase)	*narK2, G2, H2, G3, I3, H, G;* SCO0212, SCO0922 to SCO0924
Cell envelope biosynthesis	SCO6021-SCO6025 (partially)	SCO4873-SCO4881 (including *neuAB*)
Oxidative Stress and Iron metabolim	*cchFEB, trxC, ahpC, rsrA*	*cpeB,* SCO2114, SCO0380
Nitrogen metabolism	*glnA, glnII, amtB, glnK, SCO2197, SCO2199*	
Phosphate metabolism	*phoH, SCO2533*	*pstSCAB, phoU, phoRP, phyA, phoA, phoC, phoD, pitH2,* SCO1565, SCO1968, SCO7697
Morphological differentiation	*chpC, wblA, wblC,* SCO5189, SCO5191, SCO7251, SCO7252, *bldA, bldC, bldD, bldK, bldM, cdgA, cdgB*	*sigU*
Secondary metabolism	*cdaR, scbR-scbA* *redDGHKMNPQYX* [a] *cpkAIJK* [a]	*redDGHKMNPQYX* [b] *cpkAIJK* [b]

[a]This data are based on shift-down experiments of Rodríguez-García *et al.*[4].
[b]This data are based on batch cultures studies of Allenby *et al.*[19].

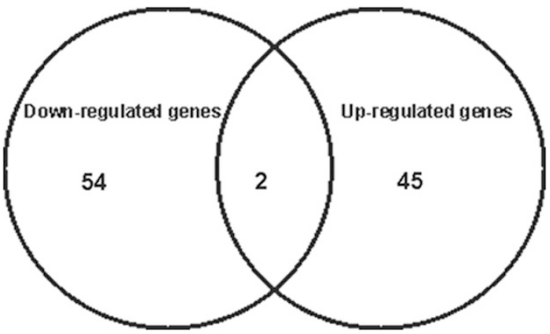

Figure 2 Summary of genes (or gene clusters, considered as a unit) that are downregulated or upregulated, respectively, by PhoP. Two genes clusters are transiently downregulated following phosphate starvation[4] and upregulated by PhoP at later times in batch cultures.[19]

the formation of reactive oxygen species (ROS). The oxidative effect is reflected in the increase expression of the catalase/peroxidase gene *cpeB*. Indeed, following phosphate starvation expression of *cpeB* increases in the wild type but not in the Δ*phoP* mutant, indicating that *cpeB* expression is PhoP dependent.[4] However, no conserved PHO boxes have been identified in its promoter, and their promoter was not enriched in the PhoP-ChIP experiment.[19] Three genes involved in the biosynthesis of the siderophore coelichelin (*cchBEF)*, which are known to be regulated by iron[28] appeared to be PhoP-repressed according to their profile in the parental strain and the mutant, although no evidence of PhoP binding has been reported.

CROSS REGULATION OF NITROGEN METABOLISM AND PhoP

Phosphate and the nitrogen sources control microbial growth in an interconnected manner. The central role in nitrogen metabolism is played by the glutamine synthetase (GS). *S. coelicolor* has five genes encoding GS-like proteins; two of them have been biochemically characterized and correspond to proteins that possess GS activity. They are *glnA,* which encodes a type I bacterial GS, and *glnII,* for an eukaryotic type GS. Nitrogen metabolism depends on the operon *amtB-glnK-glnD* coding for an ammonium transporter, a signal transmiter protein (PII) involved in the adenylation/deadenylation of the GS and a nucleotidyl transferase that modifies PII by adenylation, respectively.[29] Two regulatory genes, named *glnR* and *glnRII,* control expression of nitrogen metabolism genes. GlnR is an OmpR-family orphan response regulator with an HTH motif at the C-terminal end, which binds to GlnR-boxes located in the *glnA, glnII, amtB* and other nitrogen metabolism genes. The GlnR boxes are 22 nucleotide sequences with a conserved consensus TnAc-n6-GaAAc-n6 motif.[30] This motif has been updated by Sola-Landa *et al.*[31] The binding site of the second regulator, GlnRII, is not well known.[32] PhoP binds the *glnR* promoter, the promoters of *glnA* and *glnII* and the promoter region of the *amtB-glnK-glnD* operon. No binding of PhoP to the *glnRII* promoter was found under identical experimental conditions, both in *S. coelicolor* and *S. avermitilis;*[4,8] however, Allenby *et al.*[19] proposed, based on transcriptomic and ChIP-on-chip studies, that PhoP regulated also *glnRII* expression.

PhoP and GlnR compete to bind the same region of the *glnA* promoter, although GlnR shows higher affinity than PhoP. Binding experiments showed that PhoP binds not only the *glnA* promoter

region but also the promoters of *ureA, amtB, glnR* and other nitrogen regulated genes.[31] In summary, PhoP controls nitrogen metabolism at two different levels: (1) repressing the general regulator encoding *glnR* gene and (2) repressing specific genes of nitrogen metabolism.

The biological function of this control is to coordinate the metabolism of phosphate and nitrogen sources to get a balance of the utilization of these nutrients for the growth of *Streptomyces*.

PhoP CONTROL OF ANTIBIOTIC AND OTHER SECONDARY METABOLITES BIOSYNTHESIS: INTERPLAY WITH OTHER REGULATORY FACTORS

As indicated above, the biosynthesis of antibiotics and other secondary metabolites has been known for many years to be negatively regulated by high concentrations of inorganic phosphate in the culture medium. However, the possibility that this control is exerted directly by PhoP has been a subject of debate.[33] Not all effects of inorganic phosphate are mediated by PhoP. Inorganic phosphate regulates secondary metabolism also at the translational/posttranslation levels and through the formation of biosynthetic precursors and intermediates. Phosphate shift-down experiments[4] showed that some antibiotic gene clusters were regulated by PhoP. These include the undecylprodigiosin cluster (*red*) and to a lesser extend the actinorhodin (*act*) gene cluster. Ten genes of the *red* cluster show a transcriptional profile that indicates that they are activated by phosphate starvation and this activation is partially mediated by PhoP. In other words, these genes are not expressed during rapid growth phase and are induced following phosphate depletion, but the activation is higher in the *S. coelicolor phoP* mutant, suggesting that there is a transient repression exerted by PhoP. Allenby *et al.*[19] in batch cultures of *S. coelicolor* confirmed this transient repression of pigmented antibiotics at the time of phosphate depletion.

On the other hand, in the shift-down experiments, some secondary metabolite genes were activated soon after (before 7.5 h) phosphate starvation. These include, a type III polyketide synthetase gene (SCO7221) involved in germicidin biosynthesis[34] activated following phosphate starvation, although PhoP has a limited effect. The same occurs to several *cpk* genes,[4,9] involved in biosynthesis of the polyketide coelimycin P1,[35] which includes an oxoacyl acyl carrier protein synthetase. Expression of the *cpk* cluster for coelimycin P1 biosynthesis is dependent of the *phoP* gene,[19] although Rodríguez-García *et al.*[4] found that the *cpk* genes are transiently downregulated by PhoP. An important observation is that PhoP binds massively to three sites internal to the *cpk* cluster that corresponds to *cpkB* and *cpkC*. The role of this large amount of PhoP bound to the *cpk* cluster region is not clear. Allenby *et al.*[19] suggests that the binding of PhoP to this region may favour expression of the cluster by interacting with other regulatory proteins.

The first direct connection of the PhoP regulator with the control of antibiotic biosynthesis was made by Santos-Beneit *et al.*[36] when they proved that PhoP binds the same sequence in the *afsS* promoter as the cognate-regulator AfsR. AfsS is a small protein (63 amino acid) that activates expression of *actII-orf4* and *redD*, the cluster situated regulators of the actinorhodin and undecylprodigiosine biosynthetic clusters. PhoP was found to bind a PHO box overlapping with the sequence recognized by AfsR.[36,37] Both PhoP and AfsR are positive regulators and compete for this sequence. Indeed, the affinity of AfsR for this PHO box is higher than that of PhoP, and therefore, PhoP interferes with optimal AfsR-mediated *afsS* expression. Therefore, PhoP mediates actinorhodin and undecylprodigiosine formation through the intermediation of AfsS. Additionally, PhoP interacts with other regulatory factors (see below) and therefore there is an interplay of several regulators in the control of secondary metabolites biosynthesis.

Close to the *cpk* cluster are the divergently transcribed *scbR-scbA* genes whose intergenic region is a target for PhoP. ScbR has an important role in expression of γ-butyrolactone controlled genes, particularly the γ-butyrolactone synthase encoding gene *scbA*, and *cpkO*, encoding a pathway-specific regulator situated gene in the *cpk* cluster that is controlled by the γ-butyrolactone system. The *scbA* gene is downregulated by PhoP[19] and therefore, PhoP indirectly affects formation of γ-butyrolactones and the coelymicin P1 regulator CpkO.

PhoP controls directly other cluster situated regulatory genes, as *cdaR*. This gene is located in the cluster for the calcium-dependent antibiotic, CDA, and has been observed to be repressed by PhoP. This is the first example of a gene located in an antibiotic gene cluster regulated directly by PhoP. In contrast, a transport gene (SCP3216) in this same cluster is activated by PhoP, suggesting that there is a complex balance in the expression of the *cda* biosynthesis and secretion genes.

In summary, the transcriptomic information available indicates that some clusters for secondary metabolites formation are PhoP activated following phosphate starvation, while others are transiently repressed by PhoP.

CELLULAR DIFFERENTIATION

The *phoP* mutant showed differential expression of some morphological differentiation and development genes. Eight genes involved in cellular differentiation were found to be regulated by phosphorylated PhoP.[4] They include *sigU*,[38] encoding a sigma factor, and. the *chpC* gene for chaplin C, a protein involved in aerial mycelium formation.[39]

Several developmental genes, which are involved in aerial mycelium formation and sporulation,[40] particularly *bldA, bldC*, *bldD*, *bldK* and *bldM* are PhoP regulated.[4] PhoP binds to the promoters of these genes and repress their expression under conditions of phosphate limitation. This was confirmed also using ChIP-on-chip experiments.[19] Furthermore, *bldA* encodes a $tRNA^{leu}$ required for the expression of the rare TTA codon. The *bldA* gene is essential for the expression of *actII-orf4, redD* and several other regulatory genes that contains this codon, including some polyketide and non-ribosomal peptide biosynthetic genes. This observation indicates that PhoP controls indirectly antibiotic biosynthesis. In summary, PhoP in response to phosphate starvation represses many developmental genes and target regulatory genes, and therefore, indirectly, regulates antibiotic production in *S. coelicolor*.

BINDING OF TWO REGULATORY PROTEINS TO CLOSE POSITIONS IN THE PROMOTERS: CHROMATIN IMMUNOPRECIPITATION MAY TRAP PhoP WITH OTHER REGULATORY PROTEINS

Most of the initially characterized genes regulated by PhoP were related to the control of primary metabolism, particularly scavenging and assimilation of phosphate, and nitrogen sources transport and metabolism. More recently, Allenby *et al.*[19] have studied the PhoP binding *in vivo* by using the ChIP-on-chip technology. This technology which uses immunoprecipitation of DNA and associated proteins allows the identification of DNA regions that bind PhoP. Using ChIP-on-chip these authors identified a large number of sequences putatively regulated by PhoP, confirming most of the genes previously described[17] (Table 1).

As indicated above there is increasing evidence that some DNA regions contain sequences closely located to each other, for the binding of two or more different regulatory proteins, for example,

the genes for nitrogen metabolism frequently contain binding sequences for GlnR and PhoP, and the sequence-binding AfsR overlaps with a PhoP-binding site. Moreover, the oxidative phosphorylation genes contain sequences that are recognized by both, PhoP and the Rex regulator.[26] Similarly, in chitin metabolism, a regulatory protein, DasR, binds to promoter regions involved in N-acetylglucosamine metabolism that are also regulated by PhoP.[41] The interaction of regulatory proteins may estabilize the binding of PhoP to weakly conserved PHO boxes and explains why there are so many PhoP-binding regions detected in the DNA by the ChIP-on-chip technique.

AVERMECTIN AND THE AVERMECTIN GENE CLUSTER IN *Streptomyces avermitilis*

Avermectin is a 16-membered pentacyclic lactone type I polyketide antibiotic discovered by the group led by Prof. Satoshi Omura.[42] This compound is a very potent antihelmintic agent, active against nematode and arthropod parasites, with few side effects for the host.[43]

The avermectin producing strain, *S. avermitilis,* has a genome of 9.0 Mb that includes gene clusters for several secondary metabolites.[44] Genes for avermectin biosynthesis (*ave*) are clustered in a 90 kb DNA region. This region includes: (1) four large ORFs encoding the avermectin multifunctional polyketide synthase, required to synthesize the avermectin aglycon, (2) several genes for aglycon modifications (ketoreductases, SAM-dependent methyltransferases) and (3) seven genes for dTDP-oleandrose biosynthesis and transfer to the aglycon.[45] A cluster situated LAL-type regulatory gene, *aveR*, encodes a positive regulator essential for transcription of all *ave* genes, including the post-polyketide modification genes.[46] Deletion of *aveR* or the AveR C-terminal HTH domain, results in lack of avermectin production, but this mutant overproduces the unrelated macrolide oligomycin. ChIP-on-chip experiments and promoter activity assays showed that the *ave* genes, for avermectin, and the *olm* genes, for oligomycin biosynthesis, were targets of AveR, leading to positive and negative regulation of these clusters, respectively.[47]

THE *phoRP-phoU* GENE CLUSTER IN *S. avermitilis*

The arrangement of the *phoRP-phoU* gene cluster in *S. avermitilis* is identical to that of *S. coelicolor.* Both *phoRP* genes are transcribed divergently from *phoU.* The PhoR protein has 425 amino acids and a molecular weight of 45.4 kDa, whereas the response regulator PhoP is a small protein of 223 amino acids and a deduced molecular weight of 24.7 kDa.

The PhoR sensor kinase and the PhoP protein of *S. avermitilis* are 89 and 98% identical in amino acids, respectively, to those of *S. coelicolor.*[2,44] The extremely high conservation of PhoR, and particularly of PhoP, and the identical organization of the *phoRP-phoU* genes in *S. avermitilis, S. coelicolor*, *S. lividans* and in the sequenced genomes of other *Streptomyces,* indicates that these gene clusters have a very important role controlling the metabolism in *Streptomyces.* To characterize the role of PhoP in *S. avermitilis,* Yang *et al.*[8] obtained a *phoP*-deleted mutant and validated this mutant in complementation studies.

RT-PCR expression studies in *S. avermitilis* indicated that four key genes of the *pho* regulon, namely *phoR, phoP, phoU* and *pstS,* are clearly regulated by the phosphate availability in the culture medium. These genes are highly expressed in phosphate depleted medium but are poorly expressed in phosphate supplemented medium. Purified recombinant GST-PhoP protein was found to bind efficiently the promoters of *phoPR, phoU* and *pstS* genes. Footprinting studies performed with the *phoRP-phoU* bidirectional promoter region revealed that there are four direct repeated units (DRus) that form two complete PHO boxes. This repeated region was protected by PhoP, and indeed, up to four shifted bands were observed in gel retardation assays. The formation of up to four shifting bands suggest that there is a cooperative binding of more PhoP subunits, perhaps to less conserved sequences in the proximity.

Electrophoretic mobility shift studies revealed that PhoP binds also to promoters of several genes involved in nitrogen metabolism in *S. avermitilis*. These include *glnR, glnA* and *glnII* but not the second regulatory gene *glnRII*. These finding fully agree with the observations reported in *S. coelicolor*[48] and confirm that PhoP coordinates phosphate and nitrogen metabolism in *S. avermitilis.* PhoP in *S. avermitilis* also regulates several differentiation genes, including *bldC, bldD* and *bldM* as also observed in *S. coelicolor.*[8]

NEW BIOINFORMATICS ANALYSIS OF THE *pho* REGULON IN *S. avermitilis*

In order to take advantage of the knowledge gained on the *S. coelicolor* PhoP regulation, we performed new bioinformatic studies to identify and analyze possible PhoP-binding sites in the genome of *S. avermitilis.*

The program matrix-scan[49] (www.rsat.eu) and the version 2 of *S. coelicolor* PhoP-binding sites model[31] were used to find candidate sites in the *S. avermitilis* genome. The model 2 was used as a count matrix of 22 nt of length corresponding to one PHO box (2 DRus). In our study, the most highly conserved PHO box located in the *pstS* promoter (there are two putative boxes in its promoter region) had a weight of 13.6, and the two boxes of the *phoR-phoU* intergenic region had weight values of 9.8 and 3.1, respectively (see Table 2). A large number of sequences (25667) showed a weight value higher than 1. In order to find possible PHO boxes, the weight data were evaluated together with the information on the location of the candidate sequences (strand, distance to the translation start site), the experimental results of Yang *et al.*[8] and the published information for the orthologous genes (KEGG orthology data used) of *S. coelicolor.* The following five filtering steps were conducted: (1) sequences showing weight values equal or higher than 10, located in genes with known *S. coelicolor* orthologue, which have experimental determination of the PhoP binding site (for example, *phoD*); (2) the same criteria as the previous step, but binding evidence limited to ChIP-on-chip results (only selected the sequence of *terA*); (3) the sequence of *galE6* was selected based on its high weight, although the rest of sequences with weight value equal or higher than 10 did not have a known orthologue in *S. coelicolor*; (4) sequences with weight values lower than 10 but with an orthologue and experimental data of PhoP binding in *S. coelicolor* (for example, *pitH2*); (5) finally, the sequences not picked up in the previous steps, but with experimental results of PhoP binding in *S. avermitilis.*[8]

The SAV1 PhoP biding site of *S. avermitilis*

From the above filtering steps, 35 sequences of 22 nt (2 DRus) were selected, corresponding to 29 genes (Table 2). The 11 nt repeated sequences were aligned and using the *weblogo* server (http://weblogo.berkeley.edu/) a logo of *S. avermitilis* PhoP-binding site was obtained. As depicted in Figure 1, the sequence logos for the *S. coelicolor* and *S. avermitilis* PhoP-binding sites are similar. The consensus sequences are the same in both species. The conservation of the sequences at each nucleotide position is indicated by the height of the letter stack. Thus, there are only minor differences between the logos of both *Streptomyces* species.

Table 2 Representative *S. avermitilis* genes containing high score PHO boxes in their promoter regions

Gene	Orthologous		Product	Strand	Start	End	Weight (scoe)	Weight (save)	Sequence	Evidence Save7859	Evidence Scoe
Phosphate metabolism											
phoU'	SAV3974	SCO4228	*phoU* Phosphate transport regulator	D	−164	−143	9.8	8	CGTCGCCCAGAGTTCACCTGGG	abd	abcd
phoU''				D	−142	−121	3.1	3.4	AGCTAGTAATGGTTCATTTGGG	abd	abcd
*phoA**	SAV5915	SCO2286	*phoA* Alkaline phosphatase	D	−185	−164	4	4.8	GTCTGCATGGCATTCATGGGCC		abd
phoC	SAV5971	SCO0828	*phoC* Alkaline phosphatase	D	−114	−93	10.8	11.6	GTTCACCAGGTCTTCATCCATC		
phoD1	SAV6139	SCO2068	*phoD* Secreted phospholipase	D	−46	−25	10	11.4	GTTCACCCGCTCTTCGCTCATA		abd
(D strand)											
phoD1				R	−76	−55	6.7	9	GTTCGCCCGCTCTTCGTTCGTC		abd
(R strand)											
*pitH2**	SAV6419	SCO1845	*pitH2* Low-affinity phosphate transporter	D	−71	−50	14.3	14.1	GTTCATCTTCCGTTCACCCAGG		abcd
*pstS**	SAV4072	SCO4142	*pstS* Secreted phosphate-binding protein	D	−174	−153	13.6	14.8	GTTCACCTTCCGTTCACTCTCG	a	abd
	SAV4405	SCO3790	Predicted phosphatase	D	−93	−72	14.8	14.9	GTTCACCCTCCGTTCAGCCGCA		abcd
*SAV_4405**											
glpQ3'	SAV6784	SCO1565	*glpQ1* Secreted glycerophosphodiester phosphodiesterase	D	−103	−82	3.7	3.5	GTCACCCGTGTGTTCTGCCGCA		abd
glpQ3''				D	−81	−60	4.9	3.9	GGTCTCCCCTCCGTCACTCGAG		abd
Nitrogen metabolism											
*amtB1**	SAV2651	SCO5583	*amtB* Ammonium transporter	D	−179	−158	6	7.8	CTTAGCAGGCAGTTCATGGACG		abd
glnR'	SAV4042	SCO4159	*glnR* Nitrogen regulatory protein GlnR	R	−113	−92	3.4	4.9	CATAACGTCTGGTTCCTCCTCG		abd
glnR''				R	−91	−70	5.4	7.6	GGTCGTGCACGGTTCGCTCGCC		abd
*glnA2**	SAV5997	SCO2210	*glnII* Glutamine synthetase II	D	−139	−118	9.5	10	GGTAACACGGGGTTCACATTCG	a	abcd
*glnA3**	SAV6005	SCO2198	*glnA* Glutamine synthetase I	R	−144	−123	8	7.8	GTTTCGCCGAAGTTAACCGGCT	a	abcd
*ureA**	SAV7104	SCO1236	*ureA* Urease gamma subunit	R	−194	−173	10.8	10.9	GTTAACCAGCCCTTGATCCACT		abd
Secondary metabolism (SM)											
aveR	SAV0935	NA	Avermectin biosynthetic regulator	R	−100	−79	2.1	3.3	GTGCGGGCTGGGTTGATCAGCA	ab	
afsS	SAV3805	SCO4425	*afsS* SM AfsS	D	−200	−179	10.2	9.6	GTTCATCGTTCGTTTATCGCGG	a	abcd
Differentiation											
bldC	SAV4130	SCO4091	*bldC* Transcriptional regulator BldC	R	−157	−136	3.4	6.4	CTACACCATCCGTCCAGCCGCG	a	
*bldM**	SAV4998	SCO4768	*bldM* Two-component regulator BldM	D	−174	−153	5.2	5.2	GCTCACCGCCCCGTAACTCTTT	a	
bldD	SAV6861	SCO1489	*bldD* Transcriptional regulator BldD	R	−60	−39	8.2	8.2	GGTTACGCAGCGTTACTCTTCC	a	
Miscelaneous											
terA	SAV1427	SCO1020	Tellurite resistance protein TerA	R	−187	−166	10.7	11.5	GGTCAACCGCCGGTAACCCGCC		c
SAV_1661	SAV1661	SCO6752	Transferase	R	−228	−207	13.3	12.7	GTTCACCGGCCGTTCACGGCCC		a
galE6	SAV1662	NA	UDP-glucose 4-epimerase	D	−211	−190	13.3	12.7	GTTCACCGGCCGTTCACGGCCC		
SAV_2069	SAV2069	SCO6170	Putative oxidoreductase subunit	R	−92	−71	12.8	13	GTTCATGTGCTGTTCACGCGGA		abcd
SAV_3375'	SAV3375	SCO4879	Conserved protein	D	−75	−54	7.4	8.1	GGCGATGTGCTGTTAACCCGTT		abcd
				D	−53	−32	1.5	2.4	GTTGCATTCCGGTTGGGCCGCG		
SAV_3375''											
SAV_3376'	SAV3376	SCO4878	Glycosyltransferase	D	−86	−65	4.3	5.9	GTTCACGGCTTCGCCACGCGTC		abcd
*sig47**	SAV5679	SCO2465	*hrdA* RNA polymerase sigma factor	D	−181	−160	4	5.5	CTCCACACTGGGTTCCGGTGCC		abcd

Table 2 (Continued)

Gene	*Orthologous*		*Product*	*Strand*	*Start*	*End*	*Weight (scoe)*	*Weight (save)*	*Sequence*	*Evidence Save7859*	*Evidence Scoe*
SAV_6349	SAV6349	SCO1906	Putative secreted protein	D	−96	−75	12	13.2	GTTCAACTGCCGTTCAGGCGCG		abcd
SAV_6969	SAV6969	SCO1394	Putative glycosyl hydrolase	R	−64	−43	13.7	14.1	GTTCAACTGTCGTTCACCCGTG		abcd
	SAV7142	SCO1196	Secreted protein	D	−292	−271	3.6	3.4	GGGAACACCCGGTCCACCTCTA		abd
*SAV_7142**											
SAV_7310	SAV7310	SCO0921	Conserved protein	D	−145	−124	10.4	10.2	GGTCACGCGCCGGTCAGCCGGC		a

The upstream sequences of the genes, from position −300 to +2 relative to the translation start site, were scanned. The matrix-scan algorithm and the count matrix model 2 (Scoe) or the SAV1 matrix (Save) scores each sequence as a weight value. Using the model 2 count matrix, a sequence identical to the consensus would have a value of 15.1.
An asterik (for example, *ure**) means that in the promoter region are PHO sequences close to the selected one and the binding site might use alternative sequences. A gene with two Pho boxes is indicated with single or double quotation marks (for example, *phoU* and *phoU'*).
Code for experimental data in *S. avermitilis* (Evidence Save) or *S. coelicolor* (Evidence Scoe): a) gel shift, b) footprinting, c) ChIP-on-chip, d) differential expression between the wild-type strain and the mutant.

PhoP REGULATION OF AVERMECTIN BIOSYNTHESIS

One of the more interesting observations of Yang *et al.*[8] is that *phoP* mutants overproduce avermectin but not oligomycin. This finding clearly indicates that PhoP exerts a negative effect on avermectin biosynthesis as occurs with some antibiotics in *S. lividans* and to some extent in *S. coelicolor.*[2,5]

The PhoP regulation of avermectin biosynthesis is mediated by the AveR regulator

As indicated above in the avermectin gene cluster there is a LAL-type regulatory gene, named *aveR,* that is known to control the avermectin biosynthetic genes. RT-PCR comparative studies between the wild-type strain and the *S. avermitilis* Δ*phoP* mutant indicated that expression of *aveR* is clearly higher in the mutant than in the parental strain at two days of incubation, although the differences were lower at 6 days. This indicates that PhoP represses the expression of the regulatory gene *aveR* at early times in the fermentation, when the transcripts for *aveR* and other *ave* genes are being formed. The PhoP regulation of *aveR* expression was confirmed by gel retardation studies and footprinting analysis. These studies revealed a clear single shifted band when PhoP was added to a 477 nucleotides DNA fragment corresponding to the promoter and upstream region of *aveR* gene. The footprinting revealed a 25 nucleotides protected region that includes two DRus of 11 nucleotides each, one of which is clearly conserved with respect to the consensus sequence and the other (an extension DRu) is less conserved.[8]

The PHO boxes are situated in positions +21 to +43 downstream of the transcription start site of *aveR*. This finding indicates that binding of PhoP to the promoter region prevents reading by the RNA polymerase.[21]

The *afsS* gene, which is well known to be recognized by PhoP in *S. coelicolor,* affects avermectin biosynthesis in *S. avermitilis*[8] and this suggests that one of the indirect targets of PhoP that controls avermectin biosynthesis is the PhoP regulation of *afsS*. Indeed, the new bioinformatic analysis (Table 2) reveals that there is a well conserved PHO box in the *S. avermitilis afsS* promoter that overlaps with the AfsR binding site.

CONCLUSIONS

In summary, the regulation of primary metabolism and also avermectin biosynthesis in *S. avermitilis* follows a similar pattern to that described in detail in *S. coelicolor.*[17,19] But, on the other hand, there are some differences in the pleiotropic regulatory genes that affect avermectin biosynthesis in *S. avermitilis* as compared to those that affect pigmented antibiotics in *S. coelicolor*. One of the most important observations is the finding that expression of the cluster situated regulatory gene *aveR* is directly regulated by binding of PhoP. Although very little information is available in clusters encoding antibiotics in other *Streptomyces* species, it is likely that other cluster situated regulators, and perhaps other types of pleiotropic regulators, are directly recognized by PhoP, as occurs in *S. avermitilis*.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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