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Diverse control of metabolism and other cellular processes in *Streptomyces coelicolor* by the PhoP transcription factor: genome-wide identification of *in vivo* targets

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ABSTRACT

Streptomycetes sense and respond to the stress of phosphate starvation via the two-component PhoR–PhoP signal transduction system. To identify the *in vivo* targets of PhoP we have undertaken a chromatin-immunoprecipitation-on-microarray analysis of wild-type and *phoP* mutant cultures and, in parallel, have quantified their transcriptomes. Most (ca. 80%) of the previously *in vitro* characterized PhoP targets were identified in this study among several hundred other putative novel PhoP targets. In addition to activating genes for phosphate scavenging systems PhoP was shown to target two gene clusters for cell wall/extracellular polymer biosynthesis. Furthermore PhoP was found to repress an unprecedented range of pathways upon entering phosphate limitation including nitrogen assimilation, oxidative phosphorylation, nucleotide biosynthesis and glycogen catabolism. Moreover, PhoP was shown to target many key genes involved in antibiotic production and morphological differentiation, including *afsS*, *atrA*, *bldA*, *bldC*, *bldD*, *bldK*, *bldM, cdaR*, *cdgA, cdgB* and *scbR-scbA*. Intriguingly, in the PhoP-dependent *cpk* polyketide gene cluster, PhoP accumulates substantially at three specific sites within the giant polyketide synthase-encoding genes. This study suggests that, following phosphate limitation, *Streptomyces coelicolor* PhoP functions as a 'master' regulator, suppressing central metabolism, secondary metabolism and developmental pathways until sufficient phosphate is salvaged to support further growth and, ultimately, morphological development.

INTRODUCTION

Phosphate (P_i) is an essential constituent of all organisms and for soil-dwelling microorganisms P_i concentrations in the environment are often growth-limiting. Microorganisms such as *Streptomyces* and *Bacillus* species are often likely to experience phosphate starvation and have developed complex systems for P_i recovery and storage. Prominent among the cell's response is the induction of alkaline phosphatases (APases) and high-affinity phosphate-specific transporters that co-operatively recover phosphate from organic sources and transport it into the cell.

In *Streptomyces* species genes involved with the recovery of phosphate are controlled by the PhoR–PhoP (PhoRP) two-component signal transduction system (1,2); when the concentration of inorganic phosphate drops below a certain level ($[P_i] \leq 0.1$ mM) the PhoP response regulator is activated by its cognate sensor-kinase, PhoR. Under P_i starvation conditions PhoR phosphorylates PhoP, and PhoP∼P is known, from previous studies, to bind to 26 promoter regions in the *Streptomyces coelicolor* genome, which control expression ca. 40 genes, comprising the PhoP regulon (Supplementary Table S1). The binding of PhoP∼P causes the activation or repression of

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PhoP regulon genes and is generally considered to be mediated by the binding of PhoP~P to relatively weakly conserved 'direct repeat upstream sequences' (DRus) of 11 nt (3). For efficient binding at promoters at least two DRus are considered to be required; deletion of a single repeat from the core binding region severely reduces PhoP binding and transcriptional activation *in vitro* (3). The PhoP consensus sequence has been found to be repeated as little as twice for efficient binding (e.g. *pstS* promoter) or as many as six times (e.g. *phoD* promoter) (3–5).

Streptomycetes are Gram-positive filamentous bacteria which undergo a complex process of differentiation to form branching mycelium, aerial hyphae and spores. They produce a diverse array of bioactive secondary metabolites and as a result of this remarkable metabolic diversity streptomycetes are of major interest to the pharmaceutical industry for fermentation of natural products such as antibiotics, immunosuppressants, anti-cancer agents and many other bioactive compounds and proteins (6–8). The production of secondary metabolites generally occurs on transition to stationary growth phase and is usually provoked by physico-chemical stresses and/or nutritional limitations. High phosphate concentrations are known to suppress the production of many secondary metabolites, actinorhodin for example (9). The control of actinorhodin (*act*) and prodiginine (*red*) biosynthesis in *Streptomyces lividans* is influenced by phosphate concentration, mediated at least in part by the two-component *phoR–phoP* system; PhoRP-null mutants over-express the *act* and *red* genes (1). Notably, in *Streptomyces natalensis*, a producer of the antifungal compound pimaricin, even low P_i concentrations abolish pimaricin production (10). There are no obvious PhoP DRu elements in the promoter regions of genes that encode specific transcriptional regulators (e.g. *actII-orf4* and *redZ*) of antibiotic gene clusters and PhoP has not been shown to bind to such promoters (11). However, it has recently been shown from *in vitro* studies, that PhoP binds to the promoter region of *afsS*, a global regulator of antibiotic pathways in *S. coelicolor* (12). This was the first demonstration of a direct link between PhoP and an antibiotic regulatory system. Furthermore, a recent study has demonstrated that *S. coelicolor* PhoP binds *in vitro* to the promoter regions of *glnR* and several structural genes involved in assimilation of ammonium, including the two glutamine synthetase-encoding genes (13). The latter study revealed the broader role of PhoP in regulation of primary metabolism, demonstrating a transcriptional regulatory link between phosphate and nitrogen assimilation.

While it is becoming clear that the PhoRP system fulfils a broad role in streptomycete metabolism its primary function is traditionally considered to be to adapt to P_i limitation by inducing the production of extracellular hydrolytic enzymes and scavenging uptake systems that allow the cell to recover inorganic phosphate from organic sources. From the literature to date, PhoP is known to target at least 26 promoter regions in *S. coelicolor* (3–5,12–16) for, among others: *phoRP* (a two-component system autoregulated by PhoP), *pstSABC* (an operon encoding a high-affinity phosphate transporter for the uptake of P_i at low P_i concentrations (17,18)), *ppk* (polyphosphate kinase) (14), *phoA and phoD* (encoding APases which facilitate the recovery of inorganic phosphate (P_i) from organic sources (4) and *SCO1565* and *SCO1968* (encoding glycerophosphoryl diester phosphodiesterases involved in the hydrolysis of deacylated phospholipids) (5). The functions of several of the other known PhoP targets are not yet clear.

We have recently developed versatile high density DNA microarrays for genome-wide analysis of *S. coelicolor* (19). In the present study these arrays were exploited to assess the *in vivo* distribution of PhoP, by ChIP-on-chip analysis, across the genome under conditions of phosphate depletion and to relate the binding events to expression of the respective target genes in the wild-type and derivative *phoP* null mutant strains. This work complements and substantially extends the previous *in vitro* studies of PhoP targets by demonstrating *in vivo* binding of PhoP to most of the known targets. Importantly, the ChIP-on-chip-based approach used here has revealed an unprecedented number of novel targets for PhoP and their known, or deduced, functions demonstrate the global involvement of PhoP in the orchestration of primary and secondary metabolism in streptomycetes. Indeed the results, taken together, suggest that PhoP serves to suppress central metabolism, secondary metabolism and developmental pathways until sufficient phosphate is salvaged to support further growth and, ultimately, morphological development.

MATERIALS AND METHODS

Bacterial strains and culture conditions

Escherichia coli BL21 was used for protein over-production. *E. coli* strains were grown at 37°C in Luria-Bertani medium. Antibiotic concentrations used for plasmid selection were 100 μg/ml ampicillin, 50 μg/ml kanamycin and 50 μg/ml apramycin. *Streptomyces coelicolor* MT1110 is a plasmid-free derivative of the prototrophic wild-type strain, John Innes Stock Number 1147 (20). The *S. coelicolor phoP* null mutant used in this study is an otherwise isogenic derivative of MT1110 that contains an apramycin resistance gene inserted into the *phoP* coding sequence at codon 67 ((21); N. Cattini *et al.*, manuscript in preparation). Streptomycete strains were cultivated as described in (22).

PhoP over-expression and purification

The entire *S. coelicolor phoP* coding sequence was amplified by using the primers 5′-CGCggatccGTGCTCGTCGT CGAGGACGA and 5′-CGCgaattcCTACGGCTCGAAC TTGTAAC (where the *Bam*HI and *Eco*RI sites are shown in lowercase), cut with *Bam*HI and *Eco*RI and ligated into *Bam*HI/*Eco*RI-cut pGEX-4 T and introduced into *E. coli* BL21. The resulting glutathione-S-transferase (GST):: *phoP* fusion constructs were verified by DNA sequencing. To obtain the GST fusion proteins, *E. coli* cells were grown in 2 × YT medium at 30°C in an orbital shaker (180 rpm) to an OD_{600} of 0.6. Expression was induced with IPTG (0.1 mM final concentration) for 5 h. Cells were

harvested by centrifugation, washed twice with PBS, lysed by sonication and then mixed with Glutathione Sepharose-4B beads (Pharmacia Biotech). Proteins were eluted with 10 mM reduced glutathione (in 50 mM Tris·HCl, pH 8.0) following the manufacturer's recommendations and conserved in 40% glycerol at −80°C before use. Protein concentration was determined with the Bradford reagent (Bio-Rad). Thrombin-cleaved (liberated) PhoP was provided to Eurogentec S.A. (Liege, Belgium) for the production of polyclonal antibodies. Antibodies were purified using a Nab Protein A spin column kit (Pierce) and *in vitro* activity and specificity was confirmed by western blot analysis (data not shown).

DNA microarrays for expression and ChIP-on-chip analysis

The Sco-Chip^2-v2 array platform comprising 44 000 experimentally validated 60 mer probes, (19) was used in this study. The probes on the microarray were designed to represent coding and intergenic regions, thereby allowing both gene expression and ChIP studies on the same microarray probe set.

Chromatin immunoprecipitation

Fifty ml 2 × YT medium was inoculated with *S. coelicolor* MT1110 spores (20). After 8 h of growth at 30°C the germinated spores/mycelium were pelleted by centrifugation (10 min, 4000*g*), then resuspended in 100 µl R5 medium (22) and used to inoculate 200 ml R5 liquid medium supplemented with phosphate at a low concentration (0.009 mM KH_2PO_4). Samples were then taken from two independent flasks at 24, 30, 35 and 45 h of growth where, at each time point, 50 ml of culture was cross-linked by the addition of formaldehyde to a final concentration of 1%, and then incubated at 30°C for 30 min. The most appropriate time point for conducting the ChIP-on-chip experiments was selected after identifying, from parallel gene expression analysis, the time point representing maximal induction of known key genes of the PhoP regulon (e.g. *pstS*); this corresponded to 30 h under the growth conditions used (Supplementary Figure S1). In parallel the same ChIP procedure was conducted on cultures of the *phoP* null mutant, grown under the same conditions, as a negative control. The formaldehyde was quenched by the addition of 15 ml 3 M glycine and the cultures were incubated for 5 min at room temperature with gentle mixing. Cells were washed twice with cold phosphate-buffered saline (PBS; pH 7.4) and frozen at −80°C. Approximately 100 mg wet weight frozen cells and 1 × protease inhibitor cocktail (one tablet dissolved in 10 ml (Roche)) were added to a FastPrep tube containing glass beads. This was then shaken with a FastPrep-system for 30 s and refrozen. This process was repeated twice and finally the frozen mycelium powder was resuspended in 500 µl PBS solution. An aliquot was removed for DNA size determination; prior to electrophoresis, the sample was treated with RNase and Proteinase K and visualized on a 1% agarose gel (data not shown). Depending on the size of the DNA fragments they were further subjected to sonication, typically for 1 min (1 s on, 1 s off, 50% power; Sonics VibraCell VCX130 (Switzerland)), which sheared the DNA to 500–1000-bp fragments; size distribution of sonicated DNA samples was assessed by agarose gel electrophoresis (data not shown). Cell debris was removed by centrifugation (30 min at 16 000*g* at 4°C, conducted twice). For the immunoprecipitation 225 µl of clarified lysate was combined with 500 µl of immunoprecipitation buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5% Triton X-100) plus 1× protease inhibitor cocktail and pre-cleared for 1–3 h at 4°C with gentle agitation with 80 µl of a 50% protein A-Sepharose slurry (Sigma-Aldrich). One hundred µl purified polyclonal anti-PhoP antibodies (prepared by Eurogentec S.A.) were added to the pre-cleared sample and incubated overnight at 4°C with gentle agitation. To isolate the immunoprecipitated complexes, 100 µl 50% protein G-Sepharose slurry (washed twice in 5 µg/ml BSA) was added and incubated for 4–6 h at 4°C with gentle agitation. The protein A-Sepharose beads were washed five times (30-min incubations at 4°C with gentle agitation) with 1 ml of immunoprecipitation buffer. Beads were transferred to a fresh microfuge tube after the first wash to prevent contamination of protein/DNA that had adsorbed to the sides of the first microfuge tube. To elute the protein/DNA complexes, the beads were incubated overnight at 55°C in 240 µl elution buffer (Tris-EDTA [TE], pH 7.6, 0.2 mg/ml proteinase K, 1% sodium dodecyl sulfate). Following overnight incubation at 55°C, the samples were incubated at 65°C for 30 min to reverse formaldehyde cross-linking. The supernatant was recovered and the beads were washed with 50 µl of TE (pH 7.6) which was combined with the eluate. Two µl of 20 mg/ml glycogen mix (Sigma G-1767) was added, and the samples were extracted with an equal volume of phenol-chloroform (1:1). The DNA was precipitated with 0.1 volumes of 3 M sodium acetate (pH 4.8) and two volumes of 100% ethanol, washed with 70% ethanol and air-dried. The sample was resuspended in 40 µl of TE. A 'no PhoP antibody' control was treated as above except that water was added instead of anti-PhoP purified antibodies. It was found that enough non-specific DNA was carried through a 'no PhoP antibody' immunoprecipitation to be labelled for microarray hybridization.

Immunoprecipitated DNA and control 'no PhoP antibody' DNA were labelled with Cy3-dCTP and Cy5-dCTP, respectively, using the BioPrime kit (Invitrogen). DNA (0.01–0.1 µg) was denatured at 94°C for 3 min in 40 µl including 20 µl 2.5 × random primer mix and kept on ice. Five µl nucleotide mix (2 mM dATP, 2 mM dGTP, 2 mM dTTP, 0.5 mM dCTP), 3.75 µl Cy3/Cy5-dCTP (Perkin Elmer) and 1.5 µl Klenow fragment were added and the reaction was incubated at 37°C overnight. The labelled DNA was purified using the MinElute PCR purification kit (Qiagen) and the incorporated Cy3/Cy5-dCTP was quantified with the NanoDrop ND-1000 spectrophotometer.

Hybridizations were carried out using 10–40 pmol of Cy3 labelled immunoprecipitated DNA co-hybridized

with the same amount of 'no PhoP antibody' Cy5 labelled control mock IP DNA in 120 µl hybridization buffer (19). To control for Cy-dye bias the hybridization was repeated with the same IP DNA samples labelled in the opposite dye orientation. This was conducted for two biological replicates. The arrays were then washed with Wash 1 [6 × standard saline phosphate/EDTA (0.18 M NaCl/10 mM phosphate, pH 7.4/1 mM EDTA) (SSPE)/0.005% *N*-lauryl sarcosine] and Wash 2 (0.06% SSPE/0.18% polyethylene glycol 200), both for 5 min at room temperature. The ChIP-on-chip data are deposited with ArrayExpress (Accession Number: E-MAXD-50).

Gene expression analysis

Streptomyces coelicolor MT1110 and *phoP* null mutant cultures were prepared under identical conditions to those used for the ChIP analysis. Samples were taken at 24, 30, 35 and 45 h. RNA isolation and labelling was as described in http://www.surrey.ac.uk/fhms/microarrays/Downloads/Protocols/index.htm. RNA quality and integrity were assessed using an Agilent Bioanalyzer and a selection of northern blot analyses. Briefly, fluorescently labelled cDNA was produced from 10–15 µg of RNA using random primers and Superscript II reverse transcriptase (Invitrogen) incorporating Cy3-dCTP (Amersham). A genomic reference DNA sample was used for all gene expression microarray hybridizations, here fluorescently labelled gDNA was labelled with Cy5-dCTP (Amersham) using random primers, extended with Klenow fragment of DNA polymerase (Invitrogen), and purified using the Qiagen MinElute columns. 50 pmol of labelled cDNA and 25 pmol of *S. coelicolor* M145 labelled gDNA was hybridized to Sco-Chip^2-v2 IJISS arrays as described previously (19). The gene expression data are deposited with ArrayExpress (Accession Number: E-MAXD-51).

Microarray data processing and analysis

All microarrays (ChIP-on-chip and gene expression) were scanned at 635 nm and 535 nm with an Agilent Microarray Scanner. The resultant images were processed using Agilent Feature Extraction software (Version 9.1) with the default CGH protocol (of Feature Extraction) being applied to the ChIP-on-chip arrays and a modified gene expression protocol (such that only background correction is applied to the raw data) to the gene expression arrays. The feature extraction output files were imported into R (version 2.5.0; http://www.R-project.org) and normalized using the LIMMA package (23); global median within-array normalization followed by the 'scale' across-array normalization was applied to the $\log_2$ cDNA/gDNA ratios of the expression arrays, while only the scale across-array normalization was applied to the $\log_2$ Ab/no Ab ratios of the ChIP-on-chip arrays. Flagging of poor quality spots was conducted as described by Bucca *et al.* (19) and an individual probe was filtered out of the dataset if it did not yield a good quality spot across the respective ChIP-on-chip and (separately) gene expression datasets.

PhoP-binding enrichment sites from the filtered ChIP-on-chip data were identified by clustering probes that had a significantly (corrected P-value <0.05) different enrichment of signal in the wild-type strain compared with the *phoP*-null mutant following the same protocol defined in (19). The enrichment ratio threshold, identified by inspecting the plotted distribution of significant probes (as outlined in (19)) was set to 1.5

Probes targeting the coding regions of a gene were averaged such that each annotated protein-encoding gene of *S. coelicolor* was represented by a single value for each biological replicate of each condition (wild-type and *phoP*-null mutant, giving four values in total). This averaged dataset was then analysed for differential expression between wild-type and the *phoP* null mutant using Rank Products analysis (24) via the RankProd package in R (version 2.5.0) (http://www.R-project.org; (25,26) and by the Welch T-test, following Benjamini and Hochberg correction. Differentially expressed genes were identified, respectively, as either having a probability of false prediction (pfp) value ≤ 0.1 or a corrected P-value of <0.05.

Comparative sequence analysis of putative PhoP targets

MEME (Multiple EM for Motif Elicitation; meme.sdsc.edu/) was used to construct a position-specific weight matrix (PSWM) to derive sequence motifs from the previously known 26 PhoP target promoter regions and from the list of PhoP–ChIP-enriched putative promoter regions. Default settings were applied, except that for the latter the minimum width of the motif to search for was set to 7 nt, maximum width was set to 25 nt and the minimum number of sequences to include was set to 150. The PSWM encompasses two 7 nt DRus with a spacing of 4 nt. The *S. coelicolor* genome was scanned using the RSAT Matrix Scan tool with default settings (http://rsat.ulb.ac.be/; (27)). The predicted protein sequences of selected PhoP target genes were analysed by the iterative PSI-BLAST algorithm to identify related proteins or protein domains from the non-redundant protein databases (October 2011; http://www.ncbi.nlm.nih.gov/blast/).

RESULTS

Identification of an unprecedented range of PhoP targets in phosphate-depleted cultures of *S. coelicolor* by ChIP-on-chip analysis coupled with gene expression profiling

Previous *S. coelicolor* studies have used a combination of promoter sequence analysis, transcriptomics and proteomics to identify putative PhoP-regulated genes (3,4,15,28). In the present study we have determined *in vivo*, by ChIP-on-chip analysis, the genome-wide distribution of the *S. coelicolor* PhoP transcription factor in cultures at the point where phosphate becomes limiting. We have directly correlated the respective binding events with differences in PhoP-bound-proximal gene expression between a wild-type and a *phoP*-null mutant strain. *Streptomyces coelicolor* MT1110 and the otherwise isogenic *phoP*-null mutant derivative were grown in a phosphate-limited version of R5 medium, which facilitates

the phosphate starvation-induced entry into stationary phase (4). The concentration of phosphate was monitored throughout growth for each culture, to identify the point at which P_i was exhausted from the medium (data not shown). Samples were taken at different time points for both ChIP-on-chip and gene expression analysis as detailed in Materials and Methods. Inspection of the gene expression profiles showed that maximal induction of the *pstS* gene of the *pst* operon and of *phoR-phoP* (data not shown) occurred after ca. 30 h growth (Supplementary Figure S1). Since this time represents the stage at which PhoP∼P is likely to be most active it was selected for the ChIP-on-chip analysis to create a 'snapshot' of the *in vivo* genomic distribution of PhoP.

Overview of ChIP-on-chip and transcriptomic results

To identify DNA targets bound *specifically* by PhoP a parallel ChIP-on-chip analysis was performed using chromatin from a *phoP*-null mutant. The respective microarray probe signals derived from the wild-type cultures were divided by the probe signals obtained from the corresponding *phoP* mutant cultures; thus ChIP-enrichment peaks common to both strains would cancel each other out. From this study 640 regions of the genome were identified as significantly enriched by PhoP (Figure 1; Supplementary Table S2). Many of these significant 'regions' encompass multiple genes, grouped together by the clustering algorithm used, and it is clear that the binding of PhoP is not restricted to promoter regions; 1949 of the statistically significant PhoP–ChIP-enriched probes represented coding sequences whereas 816 probes comprised non-coding sequences. From within this dataset 417 genes were identified that had at least 2 ChIP-enriched probes within the 500 nt region centred on the start codon of the gene (Supplementary Table S3). Most of these 417 genes demonstrated differences in expression between the wild-type and *phoP* null mutant strain: based on the average values from the replicate transcriptome data 92% demonstrated at least a 25% difference, whereas 57% demonstrated at least a 50% difference in expression. Although PhoP has generally been considered to function as an activator of transcription our analysis indicates that more genes are negatively regulated, than positively, by PhoP. This is illustrated by a global comparison of wild-type versus *phoP* mutant expression levels between all genes and the 417 genes identified above (Supplementary Figure S2).

Several previous *in vitro* studies have sought to identify a consensus 'PhoP Box' sequence for PhoP-regulated genes. The 26 known target promoters were used to construct a PSWM (Supplementary Table S1) and the derived sequence logo is presented in Figure 2A. This consensus sequence has considerable degeneracy and an RSAT genome scan identified 2088 matches; approximately equal numbers of the sequence occur in protein-coding and non-coding sequences. Of the above 417 genes with PhoP-enriched probes close to the start of the gene, 100 (24%) contain a match to the PSWM (Supplementary Table S3). In a separate analysis, the 500 nt putative promoter regions of the 417 genes (comprising 333 unique regions because several are divergently transcribed) were searched for motifs using MEME and almost half (150 of 333) contained a 9 nt motif that corresponds closely to half of the PhoP Box motif (Figure 2B).

From the transcriptome analysis alone ∼570 genes were found to be differentially expressed in the wild-type strain relative to the *phoP* mutant (Supplementary Table S4). The combined results of this study are summarized genome-wide in Figure 1.

Congruence of *in vivo* ChiP-on-chip results with *in vitro* studies: PhoP-mediated control of phosphate and nitrogen assimilation

The primary purpose of this study was to build a comprehensive picture of the *in vivo* targets of PhoP and to determine, in each case, whether the binding event had a positive or negative influence on expression of adjacent genes. Such studies will ultimately enable the construction of a global regulatory network for PhoP action. It was, therefore, important to first demonstrate that our ChIP-on-chip/transcriptome approach identified previously known PhoP target genes. Several recent studies in *S. coelicolor* have identified genes regulated by PhoP, for example (3,15,28). The ChIP-on-chip data generated in the present study did indeed identify the majority (20 out of 26) of the known promoter regions targeted by PhoP (Supplementary Table S1) and the parallel transcriptome data confirmed whether PhoP functioned as an activator or repressor of the respective target genes/operons at the stage at which the cultures entered phosphate limitation. Data for a selection of previously known PhoP targets is plotted in Supplementary Figure S3. The best known PhoP target is the *pstSCAB* operon and it is noted here that PhoP appears to bind upstream of the internal *pstA* gene in addition to the *pstS* promoter region (Supplementary Figure S3A). We also note that PhoP activates its own transcription by binding to the promoter region of its cognate partner gene, *phoR* (Supplementary Figure S3B). In general, under conditions of phosphate limitation PhoP activates genes required for phosphate liberation, scavenging and assimilation while in contrast PhoP represses genes required for nitrogen assimilation; PhoP directly represses the regulatory gene, *glnR* and the key structural genes, confirming the recent *in vitro* observations (13) (Supplementary Figure S3). The ChIP analysis also identified additional putative PhoP target genes involved, or potentially involved, in regulation of nitrogen metabolism, including *glnRII* and *SCO0255* (Supplementary Figure S4); the latter encodes a NtrC-like regulatory protein is a known target of GlnR (29).

From the present study, it is deduced that PhoP binds to several hundred genomic regions in addition to the 26 previously known promoter regions; the experimental design used here controls for PhoP non-specific enrichment by factoring in parallel experiments with a *phoP* null mutant. A striking finding of this study is that the majority of these additional putative PhoP targets are *negatively* regulated by PhoP, rather than activated. Some of the additional key pathways targeted by PhoP are considered below.

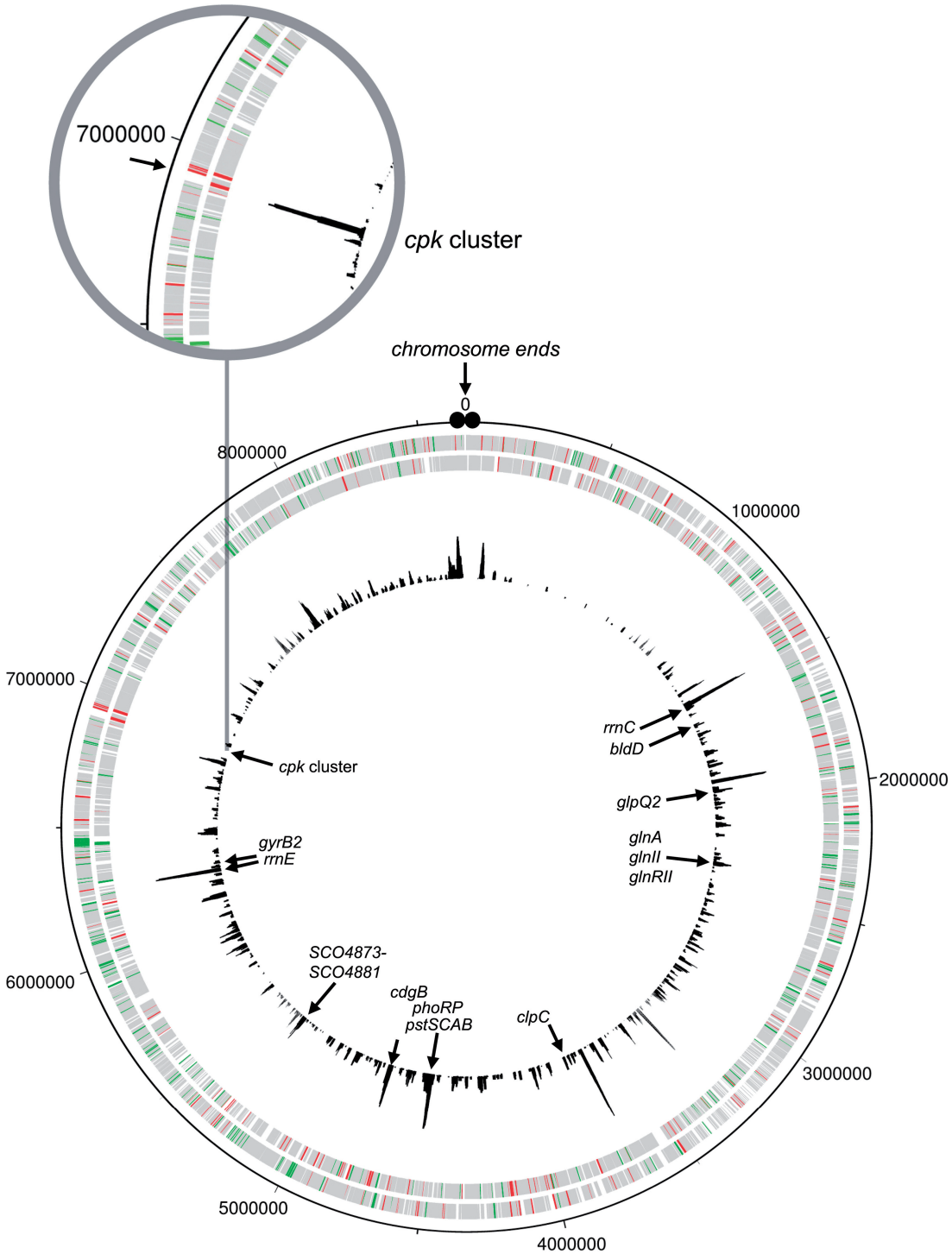

Figure 1. Summary of genomic distribution of PhoP–ChIP-enriched probes and differential expression of genes in wild-type and *phoP* mutant strain. Key to tracks from the outer circle in: track 1, all protein coding genes encoded in the 5′→ 3′ DNA strand; track 2, all protein coding genes encoded on the opposite DNA strand. Genes not significantly differentially expressed are coloured grey; genes that are significantly down-regulated in a *phoP* null mutant are coloured red and those significantly up-regulated in the *phoP* mutant are coloured green (see Supplementary Table S4 for gene list). Track 3 indicates probes (and enrichment ratio) that are significantly enriched by PhoP–ChIP. The zoomed-in area illustrates the highly enriched PhoP-binding regions in the *cpk* cluster, which represent by far the most enriched region of the genome; the ChIP-enriched probe data for the *cpk* probes were removed from track 3 of the main figure to avoid compression of the rest of the data. The location of ChIP peaks for selected target genes is indicated, as are the ends of the linear chromosome.

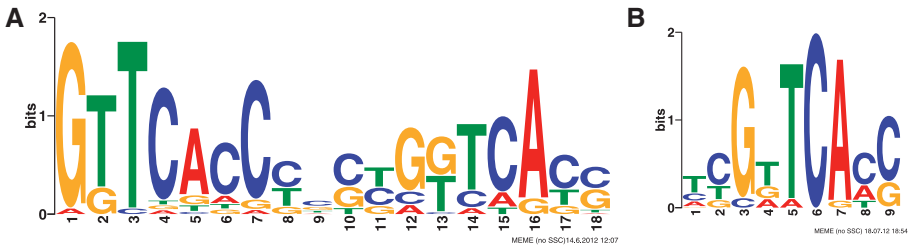

Figure 2. Sequence motifs. (**A**) The sequence logo derived from the 'PhoP box' sequences of the 26 previously identified promoters (listed in Supplementary Table S1). (**B**) The related 9 nt motif identified within the 500 nt sequences of the 417 PhoP–ChIP-enriched 5′ gene regions (listed in Supplementary Table S3); this is derived from 150 of the 333 unique promoter regions (several are divergently transcribed sequences).

PhoP-mediated negative control of oxidative phosphorylation

Genes encoding all the major components of the oxidative phosphorylation pathway are directly negatively regulated by PhoP upon entry to phosphate limiting conditions. This includes genes for the NADH dehydrogenase complex (*SCO4562–SCO4575*), the F-type ATPase (*SCO5366–SCO5374*), cytochrome C and B oxidase complexes (e.g. *SCO2148*, *SCO2151* and *SCO2155–SCO2156*) and the succinate dehydrogenase (*SCO4855–SCO4858*) (Supplementary Figure S5). It should also be noted that many of these genes are co-regulated by Rex, the redox-responsive repressor protein (30). PhoP is also observed to bind within some of the genes in these transcription units and this phenomenon is considered in the Discussion.

PhoP-mediated positive control of cell wall/extracellular polymer biosynthesis

One of the relatively few types of pathways that are observed to be positively regulated by PhoP, in addition to those for phosphate scavenging, are those considered to be responsible for the biosynthesis of alternative (phosphate-free) cell wall polymers. The ChIP-on-chip analysis identified two such gene clusters, *SCO4873–SCO4881* and *SCO6021–SCO6025* (Figure 3 and Supplementary Figure S4). Five of the nine genes in the former cluster were previously shown to be up-regulated in a *phoP* mutant (15) whereas six were recently shown to be co-ordinately up-regulated following phosphate starvation (31); PhoP binds to two promoter regions in the *SCO4873–SCO4881* cluster to activate transcription of the gene cluster (Figure 3A) and in this study eight of the genes are down-regulated in a *phoP* null mutant. The intergenic region between *SCO4873–SCO4874* displayed one of the highest PhoP-enrichment ratios in the ChIP-chip analysis. The divergently transcribed gene, *SCO4873*, encodes a putative *N*-acetylmannosamine-6-phosphate 2-epimerase, important in the production of N-acetylglucosamine-6-phosphate (GlcNAc-6-P) (15) and hence cell wall polymer biosynthesis. The *SCO4874* product is a conserved bacterial membrane protein, potentially functioning as a glycosyl transferase. *SCO4875* and *SCO4876*, respectively, encode proteins with similarity to bactoprenol glucosyltransferases and an integral membrane GtrA-family protein, a bactoprenol-linked sugar translocase; in some bacteria both proteins are involved in decoration of cell wall teichoic acids with glucose and galactose.

The second region targeted by PhoP in this gene cluster is the divergent promoter region of *SCO4878–SCO4879*. SCO4878 encodes a glycosyltransferase with similarity to the teichoic acid biosynthesis protein GgaB of *Clostridium botulinum*. All three genes in the *SCO4879–SCO4881* operon are predicted to have a role in polysaccharide biosynthesis. *SCO4879* encodes a conserved hypothetical protein related to the *E. coli* NeuE, whereas *SCO4880* (NeuA) and *SCO4881* (NeuB), respectively, display similarity to CMP-N-acetylneuraminic acid synthase and N-acetylneuraminic acid synthase.

The second putative cell wall/polysaccharide biosynthesis cluster to be targeted by PhoP is the *SCO6021–SCO6025* operon, which is conserved in the actinomycete group (Supplementary Figure S4). The three genes *SCO6021*, *SCO6022* and *SCO6023* display similarity to UDP-N-acetylglucosamine-lysosomal-enzyme N-acetylglucosamine phosphotransferase whereas *SCO6024* encodes a member of the GT1 family of glycosyl transferases. Finally, *SCO6025* contains an N-terminal domain hit to the 'GlcNAc-PI de-N-acetylase' family, an N-acetylglucosaminylphosphatidylinositol de-N-acetylase. Expression of this operon is only partially down-regulated in the *phoP* null mutant, and possibly reflects the involvement of additional factors in the control of this operon. In this context it is notable that another global regulator, DasR, is also proposed to target the promoter region of this operon (32) and this suggests that DasR controls both catabolic and anabolic pathways involving N-acetyl glucosamine.

From the above results it would be predicted that a *phoP* null mutant would lack certain alternative cell wall polymers. It is probably relevant in this context that colonies of the *S. coelicolor phoP* mutant used in this study, cultivated on agar plates, display extreme friability (data not shown).

PhoP targets key developmental processes

A striking and unprecedented observation from this study is the number of key 'developmental' genes that are targeted by PhoP. In most cases this study also provides direct evidence that PhoP acts to repress their transcription. Relatively little is known about the regulation of morphological development in *Streptomyces* (recently reviewed in (33)). Most of the genetically characterized

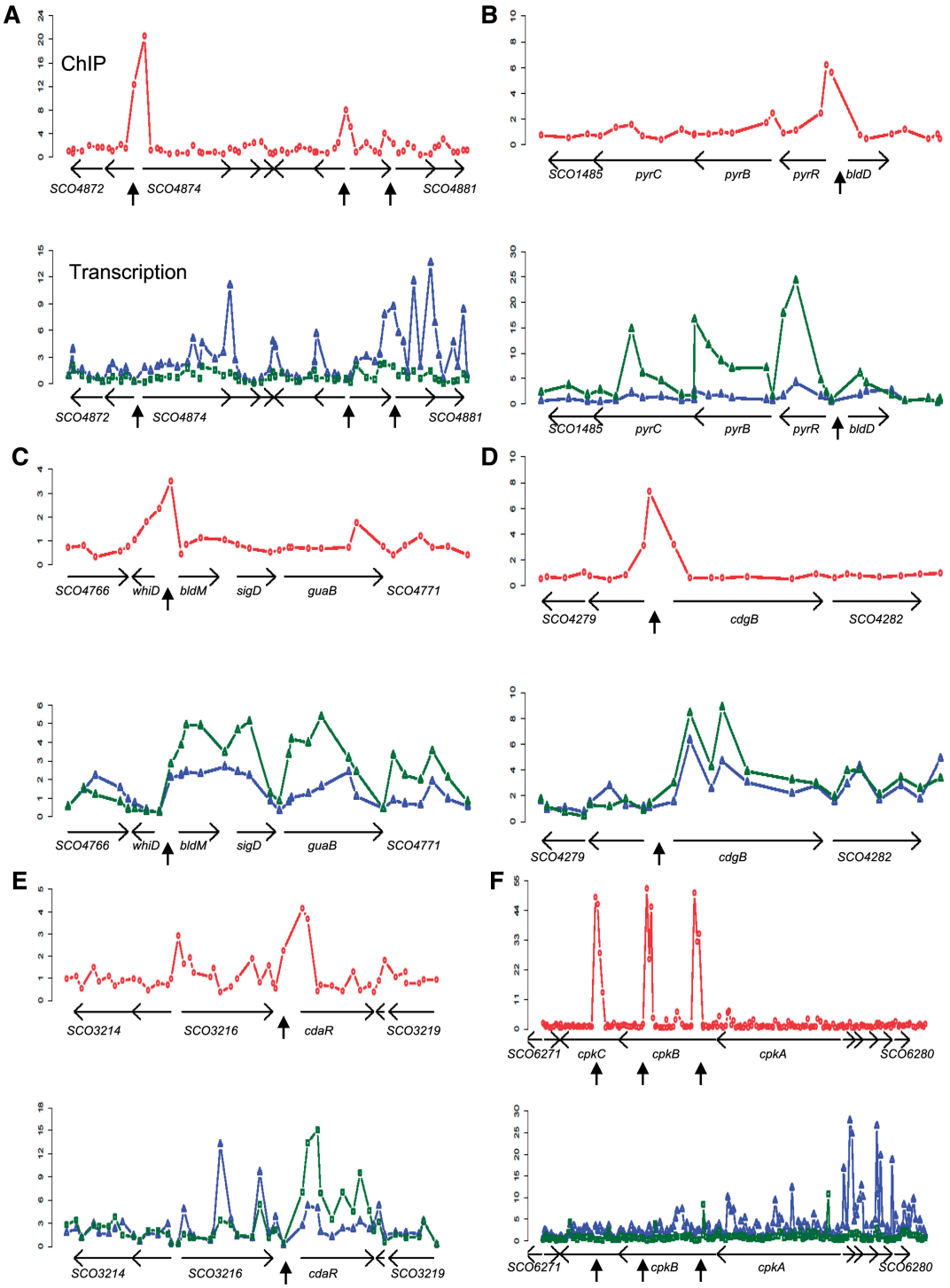

Figure 3. *In vivo* genomic distribution of PhoP and parallel measurement of adjacent gene expression, using the same microarray probe set, of selected novel PhoP targets in wild-type *S. coelicolor* and a *phoP* null mutant derivative. Two graphs are presented in each panel for each genomic region, (**A–F**). The *y*-axis of the top graph (in red) represents (i) the Cy-dye-balanced average wild-type PhoP–ChIP enrichment ratio relative to the same probe signal from the same chromatin subjected to a mock no-Ab IP **divided** by (ii) the equivalent PhoP enrichment ratio for that probe from chromatin of the *phoP* mutant [= wild-type/*phoP* mutant ChIP ratio; see Materials and Methods for more detail]; vertical arrows indicate the genomic regions deduced to be targeted by PhoP. The *x*-axis represents the genomic position of the probes and arrows represent genes; the genes at each end of the span are indicated along with specific genes of interest. Data are plotted with a custom R-script using the *S. coelicolor* genome annotation (Accession No. AL645882) for co-ordinates. For each panel the lower graph plots the derived transcript levels for each microarray probe (average [cDNA/gDNA] ratios) from the wild-type (in blue) and from the *phoP* mutant (in green). (A) PhoP-activated gene cluster *SCO4873–SCO4881* implicated in cell wall polymer biosynthesis; (B) PhoP-repressed developmental regulator *bldD* and adjacent pyrimidine biosynthesis operon (note the large scale of expression axis); (C) A cluster of PhoP-repressed developmental genes and genes required for purine biosynthesis; (D) The *cdgB* gene, encoding a diguanylate kinase; (E) PhoP-repressed *cdaR*, encoding an activator of CDA biosynthesis; (F) Massive binding of PhoP internally to three sites across two PKS genes of the PhoP-dependent *cpk* cluster.

mutants defective in development, creating a 'bald' phenotype, contain mutations in regulatory genes. This study reveals that several of the *bld* genes, *bldA*, *bldC*, *bldD*, *bldK* and *bldM* are targeted by PhoP and their expression is up-regulated in the *phoP* null mutant, indicating that PhoP represses each of these genes under conditions of phosphate limitation (e.g. Figure 3B and C). It is known that BldD also controls a large number of developmental regulation genes, including *bldA*, *bldC* and *bldM* (34). Our ChIP-on-chip data suggest that *whiH* may also represent a PhoP target (data not shown). The position of PhoP in the regulatory hierarchy allows it to exert broader control over the developmental process by repressing *bldD* expression in addition to the other regulators. The observation that the rare leucyl-tRNA-encoding *bldA* gene is negatively regulated by PhoP is relevant to the observation that *phoP* null mutants demonstrate enhanced pigmented antibiotic production (see below). PhoP is also predicted to have a broader role in that it indirectly regulates *adpA* (*bldH*), another global regulator of development and antibiotic production; *bldA* expression is required for the translation of *adpA* (35).

PhoP also directly targets the two recently identified diguanylate kinase-encoding genes, *cdgA* and *cdgB*, responsible for production of the second messenger, cyclic dimeric GMP (Figure 3D and Supplementary Figure S4). Again, both of these genes are also targeted by BldD (34,36). It is clear from the above observations that, under conditions of phosphate limitation, PhoP serves to block morphological development in *S. coelicolor*.

PhoP controls diverse secondary metabolic pathways at different levels

The regulatory networks governing antibiotic production and morphological development overlap and *bld* mutants generally do not produce antibiotics, under certain growth conditions. Thus, PhoP can partly suppress antibiotic biosynthetic pathways by repressing the above-mentioned developmental genes. For example, the *bldA* gene product is important for translation of the respective 'pathway-specific activators' for the actinorhodin and prodiginine biosynthetic gene clusters—*actII-orf4* and *redZ*; over-expression of *bldA* in the *phoP* null mutant is expected to at least partially explain the observed enhanced expression of the respective *act* and *red* gene clusters (Supplementary Figure S6). The ChIP-on-chip experiments indicated that PhoP does not bind directly to the promoters of the *actII-orf4* and *redZ* activator genes (data not shown). In contrast, our study has revealed that PhoP targets several other transcription factors that control antibiotic gene clusters, including *afsS*, *atrA, cdaR* and *scbR-scbA* (Figure 3E and Supplementary Figure S7). AfsS, the known direct target of AfsR, is a pleiotropic regulator of antibiotic production and recent independent studies demonstrated interaction of PhoP with the *afsS* promoter region (12,16). The AtrA transcription factor is known to activate transcription of *actII-orf4* (37). PhoP may have a broader impact on antibiotic production through its control of *atrA* transcription because AtrA antagonizes the role of DasR, another global regulator of antibiotic production and development (reviewed in (38)). It is not clear from our study whether the binding of PhoP to AfsS or AtrA has a positive or negative effect on expression of the respective genes because the gene expression in the wild-type and *phoP* mutant strains were not statistically significantly different; it is likely that other transcription factors and/or physiological conditions influence expression of these genes.

The *cdaR* gene and the divergently transcribed *scbR-scbA* gene pair are negatively regulated by PhoP (Figure 3E and Supplementary Figure S7). CdaR is a transcriptional activator of many genes within the *cda* gene cluster, which encodes the non-ribosomally synthesized calcium-dependent antibiotic (39,40). Expression of *cdaR* is clearly repressed by PhoP, while in contrast an adjacent gene, SCO3216, encoding a putative transporter protein, appears to positively regulated. Only a relatively small number of genes from the *cda* cluster are up-regulated in the *phoP* mutant (Supplementary Figure S6) and this suggests that regulation of the large *cda* cluster is relatively more complex than that of the clusters for the pigmented antibiotics, actinorhodin and prodiginine. PhoP also targets the *scbR-scbA* promoter region and this binding is deduced to repress *scbA* since its expression is significantly up-regulated in a *phoP* null mutant. The *scbA* gene product plays a critical role in the biosynthesis of gamma-butyrolactone signalling molecules, which positively influence pigmented antibiotic production (41,42). This deregulation is therefore also likely to contribute to the observed overproduction of pigmented antibiotics in the *phoP* null mutant.

The *scbR-scbA* gene pair is situated immediately adjacent to the large *cpk* gene cluster, which determines the biosynthesis of type I polyketide secondary metabolites (43,44). ScbR represses expression of the pathway-specific activator, CpkO, of the *cpk* cluster and this repression is reversed when ScbR binds gamma-butyrolactones (produced through the action of ScbA). Therefore, it is considered that ScbR would not repress *cpkO* in a *phoP* null mutant because *scbA* is over-expressed in such a background. Despite this, expression of the *cpk* cluster is globally down-regulated in a *phoP* null mutant. Thus, the *cpk* cluster is PhoP-*dependent*, in contrast to the other antibiotic clusters of *S. coelicolor*; indeed, maximal expression of the *cpk* cluster occurs at the point at which PhoP is considered to be activated in our study (30 h growth) (Supplementary Figure S6). Strikingly, it is observed that PhoP accumulates at exceptionally high levels at three specific sites *internal* to two of the large modular PKS-encoding genes, *cpkB* and *cpkC* (Figures 1 and 3F); this represents the region of highest enrichment by PhoP-ChIP in the genome. The possibility that this binding is responsible for activation of the *cpk* cluster is considered in the Discussion.

A broader role for PhoP in primary metabolism and other cellular processes

PhoP is observed to bind at several hundred genomic locations (Supplementary Tables S2 and S3) and, in many cases, this binding correlates with differences in the

expression of adjacent genes (identified by comparing expression in the wild-type and *phoP* mutant derivative on the same microarray platform) (Supplementary Table S4 and Supplementary Figure S2). Nucleotide biosynthesis is directly repressed by PhoP; genes for biosynthesis of purines and pyrimidines are up-regulated in a *phoP* null mutant (e.g. Figure 3). Glycogen catabolism is also repressed by PhoP as exemplified by regulation of *glgP*, encoding glycogen phosphorylase (Figure 4). Other key cellular processes are subject to repression by PhoP. This includes the ClpC chaperone (a member of the Clp/Hsp100 family of AAA+ proteins, ATPases associated with diverse cellular activities) involved in maintenance of protein integrity/protein turnover (45) and *SCO5822*, encoding a DNA gyrase subunit, important for maintenance of DNA topology (Figure 4).

Two other operons negatively targeted by PhoP include *SCO5112–SCO5116* (*bldK*) and *SCO5476–SCO5480* which both encode putative oligopeptide ABC transporter systems (Figure 4 and Supplementary Figure S8). Both are implicated in antibiotic production and development and both are induced by supplementation with S-adenosylmethionine (SAM) (e.g. (46)). It is also notable that *metK*, encoding SAM synthetase, is substantially up-regulated in a *phoP* null mutant (Supplementary Table S4). The up-regulation of the above ABC transporters and SAM synthesis are likely to also contribute to the enhanced pigmented antibiotic production observed in the *phoP* mutant. A number of genes encoding extracellular proteins of unknown function are also identified as putative direct targets of PhoP; this includes the SigU targets, *SCO2207* and *SCO2217* (47), flanking *glnII* and *glnRII* (Supplementary Figure S8).

DISCUSSION

Our analysis of the genome-wide distribution of PhoP during phosphate limitation has revealed a surprisingly diverse role for the transcription factor. Overall, the results indicate that PhoP plays two major and contrasting roles: (i) to activate pathways for recovery of phosphate from the environment (and from cellular material) and to activate cell wall polymer biosynthesis; (ii) to (transiently) shut down central metabolic pathways, (most) secondary

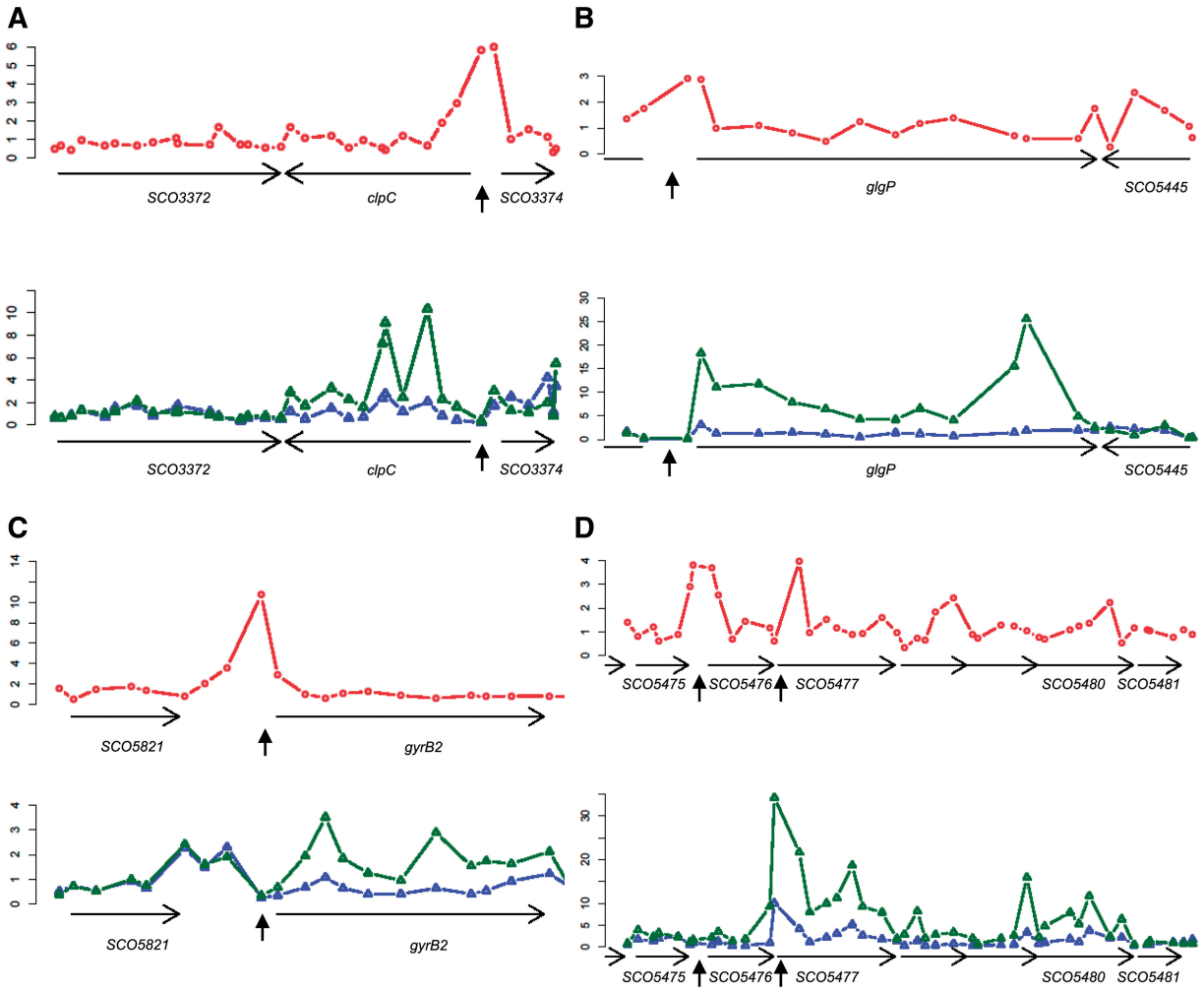

Figure 4. Distribution of PhoP-binding and differential expression of a selection of genes for diverse cellular processes in wild-type *S. coelicolor* and a *phoP* null mutant derivative. See legend to Figure 3 for detailed explanation of the respective pairs of plots. (**A**) *clpC* (protein integrity and degradation); (**B**) *glgP* (glycogen catabolism); (**C**) *gyrB2* (DNA topology); (**D**) *SCO5477–SCO5480* (ABC transporter).

metabolic pathways and the regulatory network that governs morphological differentiation. The regulatory logic underpinning these observations is straightforward to rationalize. Once *S. coelicolor* becomes limited for phosphate it is imperative for it to scavenge or liberate phosphate from the environment or from phosphate containing polymers (such as teichoic acids) if it is to re-establish growth, or initiate the process of morphological development. Since phosphate is essential for central metabolism, antibiotic production and morphogenesis it is logical for PhoP to globally repress these processes while at the same time recovering phosphate, either from environmental sources or through partial cannibalization of its own mycelium. Then, once sufficient phosphate is recovered PhoP would dissociate from its targets (presumably through dephosphorylation of PhoP) allowing resumption of growth and, ultimately, morphological development to ensue. PhoP can therefore be considered a 'conditional master regulator' providing a 'nutritional checkpoint' for morphological development. We speculate that other *S. coelicolor* nutrient-sensing transcriptional regulators, for example those sensing carbon source availability, may also exert transient global negative control over metabolism and development. The picture of PhoP-mediated control that emerges from this study is summarized in Figure 5.

The 'developmental' regulatory genes identified in this study as PhoP targets cause a 'bald' non-sporulating phenotype when mutated. Therefore, since the *phoP* null mutant does not display a 'bald' phenotype its overall influence on the developmental regulatory network must be negative.

Thus, the widespread activation and repression of diverse pathways by PhoP represents a transient co-ordinated response that allows *S. coelicolor* to essentially 'slow down' while it salvages P_i from the environment or through cannibalization of its own phosphate-containing cell wall polymers. The 'classical' well-known PhoP-activated targets are responsible for recovering phosphate while it is speculated that the two PhoP-activated putative cell wall biosynthesis gene clusters are responsible for formation of phosphate-free cell wall polymers.

It has been known for some time that mutation of *phoP* can lead to early production and over-production of antibiotics (1). Our ChIP-on-chip results reveal that this antibiotic over-production phenotype in *S. coelicolor* (at least of the two pigmented antibiotics) may be explained by alterations in the expression of several pleiotropic regulators. Firstly, the enhanced *bldA* expression (Supplementary Figure S7) is predicted to lead to enhanced translation of ActII-orf4 and RedZ-encoding mRNAs, since both contain the rare UUA codon. A concomitant increase in the level of these respective pathway-specific transcriptional activators could then lead to the enhanced actinorhodin and prodiginine cluster expression (Supplementary Figure S6). Similarly, enhancement of *afsS*, *atrA* and *scbA* expression could also contribute to the increased expression of these antibiotic clusters (Supplementary Figure S7). In addition to the above, the observed enhanced *metK* expression (encoding SAM synthetase; Supplementary Table S4) is also likely to contribute to increasing antibiotic production since

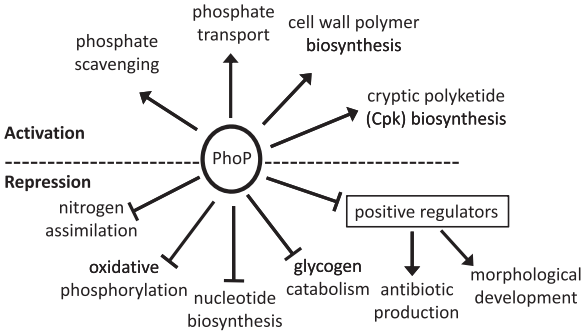

Figure 5. Overview of the breadth of pathways and processes controlled by the PhoP transcription factor when *S. coelicolor* is encounters phosphate limitation in its environment.

elevated levels of SAM are known to enhance antibiotic production (48).

The substantial binding of PhoP to three specific regions internal to the giant *cpk* structural genes represents an intriguing observation (Figure 3F). We speculate that the binding of PhoP at these sites may be responsible for the activation of expression of the PhoP-dependent *cpk* cluster. Our current view of the organization of the bacterial genome, the dynamic nature of the nucleoid coupled with topological changes and of the presence of localized 'transcription factories' has been comprehensively revised by the recent development of novel analytical tools (recently reviewed by Güell *et al.* (49)). In this context it is conceivable that PhoP co-ordinates transcription of the entire *cpk* cluster from these intragenic positions. The observation that the *cpk* cluster is apparently activated by PhoP suggests that the Cpk product(s) may fulfil a useful role under conditions of phosphate limitation. It is possible that the Cpk compounds fulfil a structural role as alternative polymers in the cell wall; the structure of the products is currently unknown.

This *in vivo* study has identified an unprecedented range of putative PhoP targets. The ChIP-on-chip analysis provided evidence for *in vivo* binding of PhoP to 20 of the 26 promoters (ca. 80%) that had been previously identified through *in vitro* studies (summarized in Supplementary Table S1) The ChIP analysis has identified additional putative PhoP targets relevant to phosphate and nitrogen metabolism: *ppk*, encoding polyphosphate kinase ((2), Supplementary Figure S3A); *glnRII*, encoding an alternative regulator of nitrogen assimilation ((29), Supplementary Figure S4), suggesting that PhoP controls both *glnR* and *glnRII*; *SCO0255*, predicted to encode a nitrogen-regulated transcription factor ((29), Supplementary Figure S4). It is of interest that PhoP also targets *SCO0255* (Supplementary Figure S4), which is itself a target of GlnR. These findings suggest that, under particular nutritional conditions, PhoP exerts over-arching control over the pathways for nitrogen assimilation. We consider that PhoP globally represses the nitrogen assimilation pathways under phosphate limiting conditions because the resulting products would be superfluous without a supply of cellular phosphate.

Six of the 26 *in vitro*-characterized PhoP-targeted promoters were not detected as PhoP-ChIP enriched in the

present study. Such 'false negatives' are relatively common in ChIP-based studies and reflect cases where the target transcription factor is shielded from the antibody in the cross-linked chromatin (e.g. by looped DNA and other proteins). It is worth noting here that perhaps the best-studied promoter region of any system—the *E. coli lac* operon promoter—was not detected as a CRP target in a comprehensive ChIP-on-chip study (50).

Many of the PhoP targets deduced from the *in vivo* analysis contain a promoter proximal sequence resembling either the consensus 'PhoP box' or a related 'half-site'; of the 417 genes with multiple PhoP-ChIP-enriched probes close to their 5′-ends, 24% have a match to the derived PSWM matrix (Supplementary Tables S1 and S3; Figure 2A) and ca. 50% contain a related 'half-site' (Figure 2B); it should be noted that our genome scanning identified more than 2000 hits to this PhoP box matrix and it is therefore ubiquitous. Indeed, the presence of a 'PhoP box' is not a good indicator of propensity for binding of PhoP *in vitro*. For example, Sola-Landa *et al*. (3) selected 20 promoter regions for *in vitro* DNA-binding analysis by PhoP. Their criteria for selection were: the promoter sequence had a match to the PhoP box consensus sequence; the adjacent gene was differentially expressed when comparing the transcriptomes of the wild-type and a *phoP* null mutant; the predicted gene product had a role relevant to phosphate metabolism. Despite the stringency of these selection criteria only 8 of the 20 promoter regions (40%) could be shown to bind PhoP in gel-shift assays (3).

We deduce that the sequence requirements for binding of PhoP to its targets are relatively complex and poorly defined. Interestingly, our sequence analysis identified a 9 nt motif that is prevalent among the PhoP-enriched promoter regions (Figure 2B). It shares similarity with half the conventional PhoP box sequence suggesting that PhoP-binding does not require a complete PhoP box; it is conceivable that binding at such sites is contingent on direct interaction with additional DNA-binding proteins. Furthermore, it should be noted that the ChIP-on-chip method cross-links neighbouring proteins to each other as well as transcription factors to their DNA targets. Therefore, the possibility cannot be excluded that some of the putative PhoP targets identified in this study result from PhoP interacting with other regulatory proteins that are bound directly to the target DNA.

Our understanding of the *in vivo* DNA-binding of transcription factors has altered dramatically over recent years and it has become clear that many native binding events will not be demonstrable by *in vitro* experiments with linear DNA fragments and single-purified transcription factors. There are several reasons for this, succinctly reviewed by Wade *et al*. (51). For example: (i) the transcription factor may need to be covalently modified (e.g. proteolytically cleaved or phosphorylated) to enable it to bind to its DNA target; (ii) The DNA may not be in a topological state appropriate for binding the transcription factor; (iii) additional accessory proteins may be required to facilitate binding of the transcription factor and/or higher order multimers of the transcription factor may be required for co-operativity in binding, circumventing the need for a full consensus sequence; (iv) the test transcription factor may require a specific ligand to facilitate binding. For these reasons *in vitro* gel-shift assays are not considered to be a useful independent approach to comprehensively 'validate' ChIP-on-chip data; the latter approach captures the native transcription factor bound to its DNA targets (along with any other interacting protein/RNA partners) within native chromatin at a particular natural physiological state. As an alternative to *in vitro* analysis, the parallel assessment of expression of all genes in the wild-type strain and in that of a mutant lacking the specific transcription factor under test, can serve to provide some verification of the ChIP-on-chip data, particularly where the RNA is isolated from the same (or replicate) cultures and assessed on the same microarray probe set, as was done in the present study.

The PhoP-ChIP analysis identified a considerable number of target sites distributed across the genome, a finding that might be expected of a globally acting transcription factor. It is, however, possible that PhoP has co-evolved a separate role in contributing to the organization of the nucleoid. In this context, not all PhoP binding events would be expected to lead to changes in expression of adjacent genes and it is envisaged that PhoP would interact with other 'global' proteins to fulfil such a role. The concept of such dual functionality of pleiotropic regulators has been reported for some time (e.g. reviewed by Wade *et al*. (51)).

The technologies are now in place to enable high-throughput mapping of the *in vivo* interactions of individual transcription factors with their genomic targets. Multiple iterations of the ChIP-chip (or ChIP-seq)/gene expression approach, such as that described in this study, will allow us to reconstruct comprehensive transcription factor regulatory networks and will reveal to what extent multiple transcription factors interact with common promoter regions to fine tune transcriptional responses. The observations that PhoP and GlnR interact with some common targets whereas PhoP and BldD share other common targets suggest that multiple transcription factor binding *in vivo* might be widespread in *S. coelicolor*. The true extent of overlap, or redundancy, of regulatory networks in *S. coelicolor* will be revealed once large numbers of transcription factors have been analysed globally by the types of approach described in this report. We are currently addressing this question by undertaking genome-wide mapping of a variety of *S. coelicolor* transcription factors.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Tables 1–4, Supplementary Figures 1–8 and Supplementary Reference [52].

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REFERENCES

1. Sola-Landa,A., Moura,R.S. and Martin,J.F. (2003) The two-component PhoR-PhoP system controls both primary metabolism and secondary metabolite biosynthesis in *Streptomyces lividans*. *Proc. Natl Acad. Sci. USA*, **100**, 6133–6138.
2. Ghorbel,S., Smirnov,A., Chouayekh,H., Sperandio,B., Esnault,C., Kormanec,J. and Virolle,M.J. (2006) Regulation of *ppk* expression and *in vivo* function of Ppk in *Streptomyces lividans* TK24. *J. Bacteriol.*, **188**, 6269–6276.
3. Sola-Landa,A., Rodriguez-Garcia,A., Apel,A.K. and Martin,J.F. (2008) Target genes and structure of the direct repeats in the DNA-binding sequences of the response regulator PhoP in *Streptomyces coelicolor*. *Nucleic Acids Res.*, **36**, 1358–1368.
4. Apel,A.K., Sola-Landa,A., Rodriguez-Garcia,A. and Martin,J.F. (2007) Phosphate control of *phoA*, *phoC* and *phoD* gene expression in *Streptomyces coelicolor* reveals significant differences in binding of PhoP to their promoter regions. *Microbiology*, **153**, 3527–3537.
5. Santos-Beneit,F., Rodriguez-Garcia,A., Apel,A.K. and Martin,J.F. (2009) Phosphate and carbon source regulation of two PhoP-dependent glycerophosphodiester phosphodiesterase genes of *Streptomyces coelicolor*. *Microbiology*, **155**, 1800–1811.
6. Hopwood,D.A. (1999) Forty years of genetics with *Streptomyces*: from i*n vivo* through *in vitro* to *in silico*. *Microbiology*, **145**, 2183–2202.
7. Hopwood,D.A. (2003) *Streptomyces* genes: from Waksman to Sanger. *J. Ind. Microbiol. Biotechnol.*, **30**, 468–471.
8. Thompson,C.J., Fink,D. and Nguyen,L.D. (2002) Principles of microbial alchemy: insights from the *Streptomyces coelicolor* genome sequence. *Genome Biol.*, **3**, 1020.
9. Bystrykh,L.V., Fernandez-Moreno,M.A., Herrema,J.K., Malpartida,F., Hopwood,D.A. and Dijkhuizen,L. (1996) Production of actinorhodin-related "blue pigments" by *Streptomyces coelicolor* A3(2). *J. Bacteriol.*, **178**, 2238–2244.
10. Mendes,M.V., Tunca,S., Anton,N., Recio,E., Sola-Landa,A., Aparicio,J.F. and Martin,J.F. (2007) The two-component *phoR-phoP* system of *Streptomyces natalensis*: inactivation or deletion of *phoP* reduces the negative phosphate regulation of pimaricin biosynthesis. *Metab. Eng.*, **9**, 217–227.
11. Martin,J.F. (2004) Phosphate control of the biosynthesis of antibiotics and other secondary metabolites is mediated by the PhoR-PhoP system: an unfinished story. *J.Bacteriol.*, **186**, 5197–5201.
12. Santos-Beneit,F., Rodriguez-Garcia,A., Sola-Landa,A. and Martin,J.F. (2009) Cross-talk between two global regulators in *Streptomyces*: PhoP and AfsR interact in the control of *afsS*, *pstS* and *phoRP* transcription. *Mol. Microbiol.*, **72**, 53–68.
13. Rodríguez-García,A., Sola-Landa,A., Apel,K., Santos-Beneit,F. and Martín,J.F. (2009) Phosphate control over nitrogen metabolism in *Streptomyces coelicolor*: direct and indirect negative control of *glnR*, *glnA*, *glnII* and *amtB* expression by the response regulator PhoP. *Nucleic Acids Res.*, **37**, 3230–3242.
14. Chouayekh,H. and Virolle,M.J. (2002) The polyphosphate kinase plays a negative role in the control of antibiotic production in *Streptomyces lividans*. *Mol. Microbiol.*, **43**, 919–930.
15. Rodriguez-Garcia,A., Barreiro,C., Santos-Beneit,F., Sola-Landa,A. and Martin,J.F. (2007) Genome-wide transcriptomic and proteomic analysis of the primary response to phosphate limitation in *Streptomyces coelicolor* M145 and in a Delta *phoP* mutant. *Proteomics*, **7**, 2410–2429.
16. Santos-Beneit,F., Rodriguez-Garcia,A. and Martin,J.F. (2011) Complex transcriptional control of the antibiotic regulator *afsS* in *Streptomyces*: PhoP and AfsR are overlapping, competitive activators. *J. Bacteriol.*, **193**, 2242–2251.
17. Allenby,N.E., O'Connor,N., Pragai,Z., Carter,N.M., Miethke,M., Engelmann,S., Hecker,M., Wipat,A., Ward,A.C. and Harwood,C.R. (2004) Post-transcriptional regulation of the *Bacillus subtilis pst* operon encoding a phosphate-specific ABC transporter. *Microbiology*, **150**, 2619–2628.
18. Diaz,M., Esteban,A., Fernandez-Abalos,J.M. and Santamaria,R.I. (2005) The high-affinity phosphate-binding protein PstS is accumulated under high fructose concentrations and mutation of the corresponding gene affects differentiation in *Streptomyces lividans*. *Microbiology*, **151**, 2583–2592.
19. Bucca,G., Laing,E., Mersinias,V., Allenby,N., Hurd,D., Holdstock,J., Brenner,V., Harrison,M. and Smith,C.P. (2009) Development and application of versatile high density microarrays for genome-wide analysis of *Streptomyces coelicolor*: characterization of the HspR regulon. *Genome Biol.*, **10**, R5.
20. Hindle,Z. and Smith,C.P. (1994) Substrate induction and catabolite repression of the *Streptomyces coelicolor* glycerol operon are mediated through the GylR protein. *Mol. Microbiol.*, **12**, 737–745.
21. Cattini,N. (2007) Phosphate regulation in *Streptomyces coelicolor* and *Streptomyces lividans*: transcriptomic analysis of *phoP* and *ppk* mutants. *Ph.D. Thesis*. University of Surrey, UK.
22. Kieser,T., Bibb,M.J., Buttner,M.J., Chater,K.F. and Hopwood,D.A. (2000) *Practical Streptomyces Genetics*. The John Innes Foundation, Norwich.
23. Smyth,G.K. and Speed,T. (2003) Normalization of cDNA microarray data. *Methods*, **31**, 265–273.
24. Breitling,R., Armengaud,P., Amtmann,A. and Herzyk,P. (2004) Rank products: a simple, yet powerful, new method to detect differentially regulated genes in replicated microarray experiments. *FEBS Lett.*, **573**, 83–92.
25. Gentleman,R.C., Carey,V.J., Bates,D.M., Bolstad,B., Dettling,M., Dudoit,S., Ellis,B., Gautier,L., Ge,Y., Gentry,J. *et al.* (2004) Bioconductor: open software development for computational biology and bioinformatics. *Genome Biol.*, **5**, R80.
26. Hong,F., Breitling,R., McEntee,C.W., Wittner,B.S., Nemhauser,J.L. and Chory,J. (2006) RankProd: a bioconductor package for detecting differentially expressed genes in meta-analysis. *Bioinformatics*, **22**, 2825–2827.
27. Turatsinze,J.V., Thomas-Chollier,M., Defrance,M. and van Helden,J. (2008) Using RSAT to scan genome sequences for transcription factor binding sites and cis-regulatory modules. *Nat. Protoc.*, **3**, 1578–1588.
28. Sola-Landa,A., Rodriguez-Garcia,A., Franco-Dominguez,E. and Martin,J.F. (2005) Binding of PhoP to promoters of phosphate-regulated genes in *Streptomyces coelicolor*: identification of PHO boxes. *Mol. Microbiol.*, **56**, 1373–1385.
29. Tiffert,Y., Supra,P., Wurm,R., Wohlleben,W., Wagner,R. and Reuther,J. (2008) The *Streptomyces coelicolor* GlnR regulon: identification of new GlnR targets and evidence for a central role of GlnR in nitrogen metabolism in actinomycetes. *Mol. Microbiol.*, **67**, 861–880.
30. Ravcheev,D.A., Li,X., Latif,H., Zengler,K., Leyn,S.A., Korostelev,Y.D., Kazakov,A.E., Novichkov,P.S., Osterman,A.L. and Rodionov,D.A. (2012) Transcriptional regulation of central carbon and energy metabolism in bacteria by redox-responsive repressor Rex. *J. Bacteriol.*, **194**, 1145–1157.
31. Nieselt,K., Battke,F., Herbig,A., Bruheim,P., Wentzel,A., Jakobsen,O.M., Sletta,H., Alam,M.T., Merlo,M.E., Moore,J. *et al.* (2010) The dynamic architecture of the metabolic switch in *Streptomyces coelicolor*. *BMC Genom.*, **11**, 10.
32. Rigali,S., Titgemeyer,F., Barends,S., Mulder,S., Thomae,A.W., Hopwood,D.A. and van Wezel,G.P. (2008) Feast or famine: the global regulator DasR links nutrient stress to antibiotic production by *Streptomyces*. *EMBO Rep.*, **9**, 670–675.

33. Flardh,K. and Buttner,M.J. (2009) *Streptomyces* morphogenetics: dissecting differentiation in a filamentous bacterium. *Nat. Rev. Microbiol.*, **7**, 36–49.
34. den Hengst,C.D., Tran,N.T., Bibb,M.J., Chandra,G., Leskiw,B.K. and Buttner,M.J. (2010) Genes essential for morphological development and antibiotic production in *Streptomyces coelicolor* are targets of BldD during vegetative growth. *Mol. Microbiol.*, **78**, 361–379.
35. Higo,A., Horinouchi,S. and Ohnishi,Y. (2011) Strict regulation of morphological differentiation and secondary metabolism by a positive feedback loop between two global regulators AdpA and BldA in *Streptomyces griseus*. *Mol. Microbiol.*, **81**, 1607–1622.
36. Tran,N.T., Den Hengst,C.D., Gomez-Escribano,J.P. and Buttner,M.J. (2011) Identification and characterization of CdgB, a diguanylate cyclase involved in developmental processes in *Streptomyces coelicolor*. *J. Bacteriol.*, **193**, 3100–3108.
37. Uguru,G.C., Stephens,K.E., Stead,J.A., Towle,J.E., Baumberg,S. and McDowall,K.J. (2005) Transcriptional activation of the pathway-specific regulator of the actinorhodin biosynthetic genes in *Streptomyces coelicolor*. *Mol. Microbiol.*, **58**, 131–150.
38. van Wezel,G.P. and McDowall,K.J. (2011) The regulation of the secondary metabolism of *Streptomyces*: new links and experimental advances. *Nat. Prod. Rep.*, **28**, 1311–1333.
39. Hojati,Z., Milne,C., Harvey,B., Gordon,L., Borg,M., Flett,F., Wilkinson,B., Sidebottom,P.J., Rudd,B.A., Hayes,M.A. *et al.* (2002) Structure, biosynthetic origin, and engineered biosynthesis of calcium-dependent antibiotics from *Streptomyces coelicolor*. *Chem. Biol.*, **9**, 1175–1187.
40. Khanin,R., Vinciotti,V., Mersinias,V., Smith,C.P. and Wit,E. (2007) Statistical reconstruction of transcription factor activity using Michaelis-Menten kinetics. *Biometrics*, **63**, 816–823.
41. Takano,E., Chakraburtty,R., Nihira,T., Yamada,Y. and Bibb,M.J. (2001) A complex role for the gamma-butyrolactone SCB1 in regulating antibiotic production in *Streptomyces coelicolor* A3(2). *Mol. Microbiol.*, **41**, 1015–1028.
42. D'Alia,D., Eggle,D., Nieselt,K., Hu,W.S., Breitling,R. and Takano,E. (2011) Deletion of the signalling molecule synthase ScbA has pleiotropic effects on secondary metabolite biosynthesis, morphological differentiation and primary metabolism in *Streptomyces coelicolor* A3(2). *Microb. Biotechnol.*, **4**, 239–251.
43. Pawlik,K., Kotowska,M., Chater,K.F., Kuczek,K. and Takano,E. (2007) A cryptic type I polyketide synthase (*cpk*) gene cluster in *Streptomyces coelicolor* A3(2). *Arch. Microbiol.*, **187**, 87–99.
44. Gottelt,M., Kol,S., Gomez-Escribano,J.P., Bibb,M. and Takano,E. (2010) Deletion of a regulatory gene within the *cpk* gene cluster reveals novel antibacterial activity in *Streptomyces coelicolor* A3(2). *Microbiology*, **156**, 2343–2353.
45. Kirstein,J., Moliere,N., Dougan,D.A. and Turgay,K. (2009) Adapting the machine: adaptor proteins for Hsp100/Clp and AAA+ proteases. *Nat. Rev. Microbiol.*, **7**, 589–599.
46. Meng,L., Yang,S., Palaniyandi,S., Lee,S., Lee,I., Kim,T. and Suh,J. (2011) Phosphoprotein affinity purification identifies proteins involved in S-adenosyl-L-methionine-induced enhancement of antibiotic production in *Streptomyces coelicolor*. *J. Antibiot.*, **64**, 97–101.
47. Gordon,N.D., Ottaviano,G.L., Connell,S.E., Tobkin,G.V., Son,C.H., Shterental,S. and Gehring,A.M. (2008) Secreted-protein response to sigma-U activity in *Streptomyces coelicolor*. *J. Bacteriol.*, **190**, 894–904.
48. Kim,D.J., Huh,J.H., Yang,Y.Y., Kang,C.M., Lee,I.H., Hyun,C.G., Hong,S.K. and Suh,J.W. (2003) Accumulation of S-adenosyl-L-methionine enhances production of actinorhodin but inhibits sporulation in *Streptomyces lividan*s TK23. *J. Bacteriol.*, **185**, 592–600.
49. Güell,M., Yus,E., Lluch-Senar,M. and Serrano,L. (2011) Bacterial transcriptomics: what is beyond the RNA horiz-ome? *Nat. Rev. Microbiol.*, **9**, 658–669.
50. Grainger,D.C., Hurd,D., Harrison,M., Holdstock,J. and Busby,S.J. (2005) Studies of the distribution of *Escherichia coli* cAMP-receptor protein and RNA polymerase along the *E. coli* chromosome. *Proc. Natl Acad. Sci. USA*, **102**, 17 693–17698.
51. Wade,J.T., Struhl,K., Busby,S.J. and Grainger,D.C. (2007) Genomic analysis of protein-DNA interactions in bacteria: insights into transcription and chromosome organization. *Mol. Microbiol.*, **65**, 21–26.
52. Santos-Beneit,F., Barriuso-Iglesias,M., Fernández-Martínez,L.T., Martínez-Castro,M., Sola-Landa,A., Rodríguez-García,A. and Martín,J.F. (2011) The RNA polymerase omega factor RpoZ is regulated by PhoP and has an important role in antibiotic biosynthesis and morphological differentiation in *Streptomyces coelicolor*. *Appl. Environ. Microbiol.*, **77**, 7586–7594.