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RESEARCH ARTICLE

Genome-wide transcriptomic and proteomic analysis of the primary response to phosphate limitation in *Streptomyces coelicolor* M145 and in a Δ*phoP* mutant

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Phosphate limitation in *Streptomyces* and in other bacteria triggers expression changes of a large number of genes. This response is mediated by the two-component PhoR–PhoP system. A *Streptomyces coelicolor* Δ*phoP* mutant (lacking *phoP*) has been obtained by gene replacement. A genome-wide analysis of the primary response to phosphate limitation using transcriptomic and proteomic studies has been made in the parental *S. coelicolor* M145 and in the Δ*phoP* mutant strains. Statistical analysis of the contrasts between the four sets of data generated (two strains under two phosphate conditions) allowed the classification of all genes into 12 types of profiles. The primary response to phosphate limitation involves upregulation of genes encoding scavenging enzymes needed to obtain phosphate from different phosphorylated organic compounds and overexpression of the high-affinity phosphate transport system *pstSCAB*. Clear interactions have been found between phosphate metabolism and expression of nitrogen-regulated genes and between phosphate and nitrate respiration genes. PhoP-dependent repressions of antibiotic biosynthesis and of the morphological differentiation genes correlated with the observed Δ*phoP* mutant phenotype. Bioinformatic analysis of the presence of PHO boxes (PhoP-binding sequences) in the upstream regions of PhoP-controlled genes were validated by binding of PhoP, as shown by electrophoretic mobility shift assays.

Keywords:
Gene expression / *phoP* mutant / Phosphate control / Transcriptome

1 Introduction

A few years ago, several authors studying in *Escherichia coli* and *Bacillus subtilis* [1–4], proposed that expression of about 40 genes belonging to the so-called PHO regulon is controlled by the phosphate concentration [5].

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Abbreviations: DRu, direct repeats unit; **FDR,** false-discovery rate; **gDNA,** genomic DNA; **VA,** vector analysis;

In different *Streptomyces* species it is well known that the biosynthetic pathways of distinct types of secondary metabolites are regulated negatively by high-phosphate concentrations [6–11]. These metabolites belong to a variety of polyketide groups, including the anthracyclines and related compounds (*e.g.* actinorhodin), the tetracyclines, macrolides and other secondary metabolites such as aminoglycosides, amino acid-derived metabolites (*e.g.* prodigionins [12] and clavulanic acid). Depletion of phosphate in *Streptomyces coelicolor* and in other *Streptomyces* species triggers a poorly known "transition phase" in which primary metabolism is reorganized and genes inducing secondary metabolism are triggered [13]. However, the cascade involved in signal transduction from phosphate sensors to transcriptional factors

interacting with phosphate-regulated promoters is still poorly understood [14]. Knowledge of the genes that are induced by phosphate limitation during the transition phase in shift-down nutritional experiments is important to understand the interaction between primary and secondary metabolism.

We reported that expression of *S. lividans* genes involved in both phosphate-scavenging (*phoA*) and secondary metabolism (actinorhodin and undecylprodigiosin biosynthesis) is mediated by the two-component *phoR–phoP* system [15]. Mutants deleted in the *phoP* gene (Δ*phoP*) or in the *phoP* and *phoR* genes (Δ*phoR–phoP*) overexpress the actinorhodin and undecylprodigiosin biosynthesis genes [15, 16]. Recently, we showed that the PhoP response regulator binds to specific sequences (PHO boxes) in the promoter regions of several phosphate-regulated genes [17]. However, a wide analysis of phosphate regulation is required for understanding the differential effect of phosphate on primary and secondary metabolism and the possible involvement of other regulatory proteins in signal transduction mechanisms of control, as described in *B. subtilis* [18].

DNA microarrays [19, 20] and proteomic studies [4, 21, 22] have become extremely useful tools for analysis of gene expression. Since DNA microarrays of *S. coelicolor* were available, it was of utmost interest to use this tool for a global analysis of gene expression in *S. coelicolor* following phosphate shift-down experiments. Phosphate control affects many genes but only part of them will be directly PhoP dependent. Therefore, it was important to study the changes in gene expression in Δ*phoP* mutants when compared with the parental strain, and to compare these changes with those observed in the proteomics studies. In this work, the integration of transcriptomic and proteomic studies has allowed us to identify 551 genes that are significantly affected by phosphate limitation, by the *phoP* deletion or both; these have been classified into 12 types of expression profiles.

2 Materials and methods

2.1 Bacterial strains and plasmids

S. coelicolor strains M145 [23], M145[pLUX-*pstS*], and INB101 (Δ*phoP*, this work) were manipulated according to standard preservation and conjugation procedures [23], except that TBO medium [24] was used to obtain spore preparations. *E. coli* DH5α was used as the general cloning host.

pLUXAR+ is a promoter-probe vector constructed from the previously described plasmid pAR933a [25]. pLUX-*pstS* (E. Franco-Domínguez, personal communication), a derivative of pLUXAR+ containing the *pstS* gene promoter was introduced by conjugation in *S. coelicolor* M145 to obtain the strain M145[pLUX-*pstS*].

A *phoP* deletion mutant, strain INB101, was constructed by PCR targeting [26]. Two primers (CAR03 and CAR04; Supporting Information, Table S3) were designed for replacement of the *phoP* coding region by the apramycin resistance cassette of pIJ773. The cosmid SCD8A [27] allowed the introduction of the mutation into the M145 strain. The *phoP* deletion in the strain INB101 was verified by Southern hybridization using internal probes to the apramycin resistance gene and to the *phoP* gene.

2.2 Culture conditions

Cultures were performed at 30°C, 220 rpm, in defined MG medium containing starch (Scharlau; 50 g/L) and glutamate (60 mM) [28]. Four hundred milliliters of MG medium in 2-L-baffled flasks were inoculated with 10^6 spores/mL for reproducible dispersed growth. Cultures containing 15 mM potassium phosphate (MG-15 medium) were used for the phosphate-replete condition. For the phosphate shift down, the cells were centrifuged (10 min at 8000 × *g*), washed with MG medium containing 0.05 mM of phosphate (MG-005), and harvested again. Half of the cells were suspended in MG-005 medium and cultivated as above.

2.3 Luciferase assay, DNA and phosphate determinations

The *luxAB* gene expression was determined in a Luminoskan luminometer (Labsystems, Helsinki). Frozen cells from 1-mL culture samples were resuspended in 1 mL of 0.9% NaCl. Fifty microliters of 1% *n*-decanal was added to 0.1 mL of resuspended cells measuring the luminescence after 15 s of elapsed time (during 20 s of integration). Measurements were performed in triplicate. DNA content in culture biomass was determined with diphenylamine [29]. The colorimetric malachite green assay was employed to measure the phosphate in culture supernatants [30].

2.4 Nucleic acid extractions

Two to eight milliliters from liquid cultures were used to extract RNA using the RNeasy Protect Bacteria kit (Qiagen cat. no. 75552) and the protocol of V. Mersinias (http://www.surrey.ac.uk/SBMS/Fgenomics/Microarrays/html/Downloads.html). Total RNA concentration and quality were checked spectrophotometrically and by agarose gel electrophoresis. Total genomic DNA (gDNA) was isolated from a stationary-phase culture following the Kirby mix procedure [23].

2.5 2-D electrophoresis

Bidimensional electrophoresis was performed using the procedure adapted to *S. coelicolor* and described by Flores *et al.* [31]. Sixty micrograms of crude protein extract was used for IEF in 18-cm precast IPG strips with linear pH gradients of 3.0–10.0 and 4.0–7.0 using an IPGphor IEF unit (Amersham). The second dimension was run in SDS-PAGE gels of 12.5% acrylamide in an Ettan Dalt apparatus

(Amersham), and gels were subsequently silver stained following an MS-compatible protocol (Amersham). Precision Plus protein Standards (BioRad) were used as size markers. Image analyses were performed with biological triplicates by using the ImageMaster™ 2D Platinum v5.0 software (Amersham).

Protein spots were excised from gels and digested with modified trypsin (Promega). Peptide mass fingerprints were analyzed with the MASCOT software [32].

2.6 Labeling and hybridization reactions: Image quantification

S. coelicolor microarrays were obtained from the Functional Genomics Laboratory of the University of Surrey (UK). These arrays (SCp40 print run) contained duplicated PCR-probes for 7083 chromosomal genes. Four biological replicates were made for each condition. The Pronto! Universal Microarray Hybridization kit (Corning, # 40026) was used for prehybridization of the slides. Labeling reactions were performed according to the recommendations described by http://www.surrey.ac.uk/SBMS/Fgenomics. In brief, 4.2 μg of total RNA was labeled with Cy3-dCTP (Amersham), using random primers and Superscript II reverse transcriptase (Invitrogen). gDNA (3 μg) was labeled with Cy5-dCTP (Amersham), from random primers extended with the Klenow fragment of DNA polymerase (Roche). Final products were purified with MinElute columns (Qiagen) and labeling efficiencies were quantified spectrophotometrically. Two hundred picomoles of Cy3-cDNA and 20 pmol of Cy5-labeled gDNA were mixed and vacuum dried. Labeled samples were resuspended in 40 μL of the Pronto! Universal Hybridization Solution (Corning), and applied on the microarray surface. Hybridizations were carried out at 42°C for 16–20 h. After hybridization, slides were washed three times, with gentle agitation, at 42°C for 10 min in solution 1 (2X SSC, 0.5% SDS). Then, the slides were transferred to solution 2 (0.5X SSC, 0.1% SDS, 0.1 mM DTT) at room temperature, and washed three times (firstly for 15 min, twice for 5 min). TIFF images generated by the Agilent DNA Microarray Scanner G2565BA were processed with Agilent's Feature Extraction Software V7.5. The WholeSpot method was used for segmentation and signal intensities for each spot were calculated as the mean of the pixel fluorescences after interquartile range rejection of outlier pixels. Local background and spatial detrend corrections were applied, as well as the surrogate algorithm.

2.7 Data preprocessing

Data preprocessing for normalization, quality labeling, and outlier detection was performed in Microsoft Excel spreadsheets as follows. Spot data were flagged with quality values from 1 to 0 (seven categories), where 1 means that all the selected Feature Extraction quality controls were passed (see Supporting Information). Medians of $\log_2$ Cy3/Cy5 intensities (spots with flag ≥ 0.9) were calculated in each print-tip block for linear normalization. Since the *S. coelicolor* strains of this work lack the SCP1 plasmid, only data from chromosomal genes were considered in the analysis. The normalized $\log_2$ of Cy3/Cy5 intensities is referred in this work as the M_g value, and is a measure of the abundance of transcripts for a particular gene with respect to its genomic copies [33, and references therein]. To evaluate the consistency of the eight gene spot M_g values in each condition (four arrays with duplicated spots), the Grubb's test was applied following the instructions of the MADSCAN manual [34], with a significance level of 0.01. The percentage of spots detected as outliers in each quality category served to assign spot weights to the data (see Supporting Information).

2.8 Statistical analysis

To assess the statistical significance of the data we used two Bioconductor software packages run in the R environment [35]. Quantile normalization of the gDNA intensities was applied by using limma (*li*near *m*odels for *m*icroarray *a*nalysis) [36, 37]. The information from within-array spot duplicates [38], and the empirical array weights [39] were taken into account in the linear models. Five contrasts between the four experimental conditions (Fig. 2) provided the respective *p*-values for each gene – reflecting the probability that a specific gene is differentially transcribed between the two conditions compared – and five *M*-values of contrasts (M_c), as the quantification of the differential transcription. The *p*-values were corrected for multiple testing (false-discovery rate, FDR). Rank products [40], unlike limma, is a nonparametric method that allowed us to confirm and complement the results of limma, with the aim of reducing the number of false negatives. This method provided another probability value, *pfp* (*p*roportion of *f*alse *p*ositives), corrected for multiple tests in an equivalent manner to the FDR algorithm. Thus, a result from a contrast was considered as statistically significant if it passed at least one of these filters: (i) limma FDR-corrected *p*-value <0.05; (ii) Rank products *pfp* value <0.05. These *h*ypothesis-testing *r*esults were summarized with the notation H_{ri}, where i refers to the contrast number. When the contrast result was not significant $H_{ri} = 0$; if the result was significant and the respective M_c was positive, then $H_{ri} = 1$; or $H_{ri} = -1$, for a significant negative M_c value. A total of 529 genes showed statistically significant results in at least one contrast, and were selected for profile classification.

2.9 Transcription profile classification

The H_r values were used to determine the transcription pattern. The contrasts 2, 4, and 5 were expected to be more meaningful because they reflect the responses to the phosphate limitation of the wild-type strain, of the mutant strain, and the difference between both responses, respectively. The combinations of the H_r values of these contrasts that

are logically self-consistent served us to define a total of 12 profiles (Fig. 3), which comprise all the possible transcription patterns that can be discriminated. To completely assign profiles to the set of 529 selected genes these approaches were used: (i) previous H_{r5} were complemented with the results of the limma test "nestedF" (FDR-corrected *p*-values), and the gene results satisfying the logical constraints were classified into profiles of quality 1 (197 genes); (ii) when H_{r5} was null and logically inconsistent with H_{r2} and H_{r4}, only the last values were taken into account, and the profiles were assigned a quality label of 2 (295 genes); (iii) the rest of the genes (37) were classified with vector analysis (VA), described by Breitling *et al.* [41], and the profiles were given a quality label of 3. The VA approach classifies the transcription responses of two different backgrounds in eight patterns (Fig. 4). The pattern scheme is coincident with the H_r-based scheme in eight profiles (AA, DD, A0, D0, 0A, 0D, AD, and DA). The profiles aA, Aa, dD, and Dd are intermediate profiles that only the H_r-based method can discriminate. The limma-calculated M_{c2} and M_{c4} values were the input for VA.

2.10 Bioinformatics search of PhoP-binding sites

The individual information theory programs makebk, encode, rseq, dalvec, ri, multiscan, and delila [42, 43] were used to identify putative target sequences of the response regulator PhoP. PhoP-binding sites are formed with a minimum of two direct repeats units (DRus), 11 nucleotides in length, and tandemly arranged (one PHO box). An individual information weight matrix was constructed following the alignment of 16 DRus. Of those DRus, six were previously described [17] and were taken from the promoters of *pstS* (two DRus) and *phoU–phoR* (four DRus). The other DRus were identified in the promoter regions of genes SCO7697 (three DRus located between positions −165 to −133, on the coding strand from the annotated translational start site), SCO1565 (three DRus, positions −81 to −49) and SCO1968 (four DRus, positions −91 to −48). This weight matrix served to compute a measure of the individual information content (Ri) of each candidate sequence. Applying this matrix, the average Ri and SD of the above 16 DRus were 5.84 and 2.77 bits, respectively; and for the whole five binding sites, were 18.68 and 2.68 bits, respectively. These four values and the Student's *t*-distribution ($\alpha = 0.05$) served to determine thresholds for DRus (Ri $>$ 1 bit), and for whole sites (Ri $>$ 10 bits). Both strands of gene sequences (positions −300 to +101), obtained from the RSA tools web server [44], were screened with the multiscan program. The models that guided the multiscan search were designed to detect two, three, or four consecutive DRu. The above thresholds were used, except for sites composed of four DRu. In these cases, as occurs in the SCO1968 gene, one intermediate DRu was allowed to have a Ri > -1.

2.11 DNA binding to gene promoters

DNA-binding analyses were performed by electrophoretic mobility shift assays (EMSA) with the GST-PhoP^{DBD} protein (fusion of GST and the DNA-binding domain), as described previously [17]. DNA fragments of the following promoter regions were amplified by PCR (gene name, start, end; positions from the translation start site): (i) SCO0034, −279, −3; (ii) SCO1565, −242, −3; (iii) SCO1968, −253, −3; (iv) SCO2532, −285, +3; (v) SCO2878, −218, +22; (vi) SCO4261, −188, +9; (vii) SCO4879, −260, +14; (viii) SCO7697, −219, +4. Primers used are listed in Table S3 of the Supporting Information.

3 Results

3.1 Phosphate shift-down experimental design

Control experiments were initially performed to define the optimal conditions to observe expression changes in *S. coelicolor* cultures after phosphate shift down. The initial cultures were carried out in MG-15 medium (15 mM phosphate; see Section 2) to minimize differences between the wild-type *S. coelicolor* M145 strain and the Δ*phoP* mutant. The time (t_0) previous to the shift down was selected at 39 h because at that point the growth was still in the exponential phase. Moreover, at this time there was already enough biomass in a 400-mL culture for RNA and protein extractions and for the inoculum of the shift-down culture. The expression of the *pstS* gene (a phosphate-regulated gene) [17, 45] was used as reporter of the phosphate-deficiency response. The *pstS* expression remained at basal levels until the phosphate in the medium decreased below 0.1 mM (Fig. 1), a similar threshold than that reported for the PHO response in *B. subtilis* [2]. We chose 7.5 h for cell collection ($t_{7.5}$) since the induction factor of the *pstS* promoter was 140 at this time and the growth was still approximately exponential (Fig. 1). The 7.5 h phosphate-deprivation time is coincident with that used by Ishige *et al.* [19] in phosphate control studies in *Corynebacterium glutamicum*. The Δ*phoP* mutant and the parental strain showed similar growth patterns in the shift-down cultures until the sampling time. Growth, measured as DNA content, was exponential until 2 h of culture, and there was no increase in DNA content from 4 h to the sampling time in both strains (Supporting Information, Fig. S1). The continuous increment of the dry weight (Fig. 1) probably is due to accumulation of glycogen or another carbon storage polymer. The average dry weight increase in the four biological replicates was 35% (with respect to the weight at t_0) for the wild type, and 36% for the Δ*phoP* mutant strain during the 7.5 h interval. Therefore, the sampling time is a trade-off between induction of PHO response and limitation of secondary changes [46].

Therefore, the four experimental conditions for RNA and protein extraction were: (i) wild type at time 0 (Wt.t_0), (ii) wild type at time 7.5 h following phosphate shift down (Wt.$t_{7.5}$),

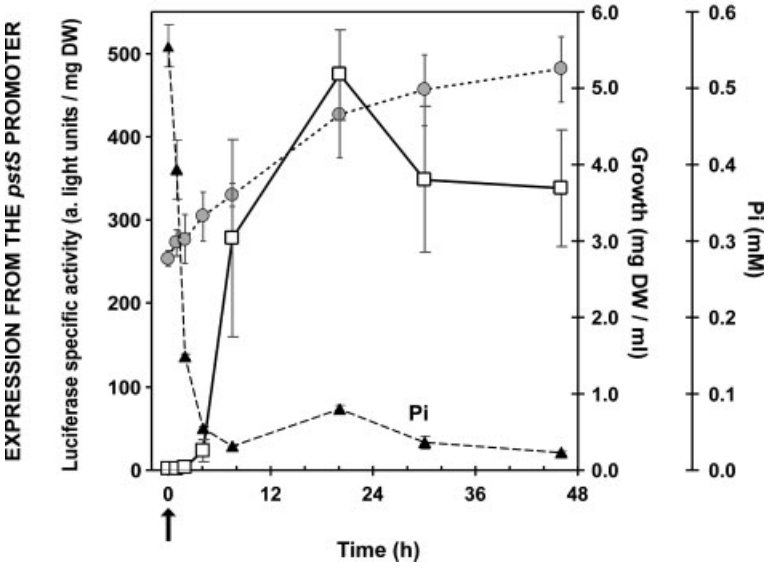

Figure 1. Induction of the *pstS* promoter after phosphate shift down. The wild-type strain carrying the *pstSp–luxAB* fusion was cultivated in MG-15 medium for 39 h. Half of the culture cells were washed (vertical arrow), and resuspended in MG-005 medium. Specific luciferase activity (squares, solid lines), growth (circles, dotted lines), and phosphate content in the medium (triangles, dashed lines) are shown as the mean of two replicates. Vertical bars show the SD. The expression of *pstS* in control phosphate-replete MG-15 medium continued at basal levels during the time course of this experiment.

(iii) Δ*phoP* mutant at time 0 (Mu.t_0), and (iv) Δ*phoP* mutant at time 7.5 h (Mu.$t_{7.5}$) (Fig. 2). The microarray approach chosen to evaluate the differential expression between those conditions involved the use of gDNA as the common reference [47, 33, and references therein]. These comparisons and contrasts are summarized in Fig. 2.

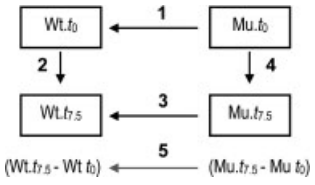

Figure 2. Linear model contrasts [36] of the microarray analysis. The experimental conditions are boxed; Wt refers to the wild type strain, and Mu, to the Δ*phoP* INB101 mutant strain. The samples were taken from cultures immediately before the shift down (t_0), and 7.5 h following shift down ($t_{7.5}$). The arrow orientation refers to the comparison between conditions. Differential transcription values M_c were obtained by subtracting the first condition M_g values from the last M_g (corresponding to the arrowhead) values, (*e.g.* Wt.$t_{7.5}$–Wt.t_0). A fifth comparison, or interaction contrast, was defined as the difference in the response to phosphate starvation between both strains (Wt.$t_{7.5}$–Wt.t_0)–(Mu.$t_{7.5}$–Mu.t_0).

3.2 A comprehensive set of transcription profiles define the wild type and the Δ*phoP* mutant primary responses to phosphate limitation

Following the statistical criteria, 529 genes showed differential transcription in at least one contrast, and were selected for further analysis. We have used the hypothesis-testing results for a logical classification of this set of genes. Considering that transcription differences between the two strains at t_0 (before phosphate shift down) are null, there are 12 transcription profiles that can be discriminated in response to phosphate limitation. A main set of eight profiles [AA, A0, AD, 0D, DD, D0, DA, and 0A; the first symbol corresponds to the wild type and the second to the Δ*phoP* mutant. A, stands for increased (*a*ugmented) expression and D for decrease; 0 indicates no change] reflect the major transcriptional responses (Fig. 3). These eight profiles were also used to summarize the pattern of protein synthesis observed in the proteomic studies (Table 2). In addition, four other profiles aA, Aa, dD, and Dd, correspond to differences between the strains when the responses are in the same direction, *i.e.* both increase or decrease, but of different intensity (small letters indicate a lower relative response).

Common regulatory inferences can be grouped in three categories of profiles. Firstly, the profiles AA and DD reflect an equal transcription response to phosphate limitation in both strains. Therefore, PhoP should not play a role in regulating those genes. More interestingly, the A0 profile indicates that expression of the genes showing this profile is PhoP dependent, since the increase in expression in the wild type following phosphate starvation is not observed in the Δ*phoP* strain. The *pstS* gene illustrates this profile since it showed the strongest response in the wild-type strain (10 times in linear scale), whereas no change in the mutant was detected (Fig. 4). That behavior fully agrees with the activity of the *pstS* promoter (Fig. 1) and with the data reported previously [17]. Other profiles that indicate a PhoP dependence were Aa, AD, 0D, and dD. In these cases, the expression of the corresponding genes was always lower in the Δ*phoP* mutant than in the wild type. On the other hand, the profiles Dd, D0, DA, 0A, and aA imply a higher expression of those genes in the Δ*phoP* mutant than that of the wild type. These higher levels can be related to a mechanism of PhoP-dependent repression.

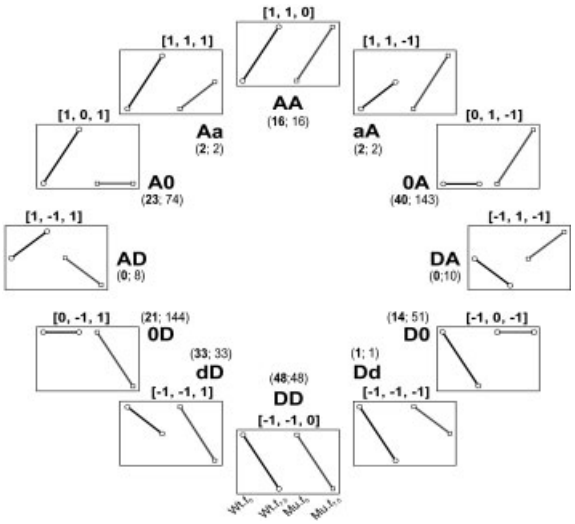

Figure 3. Transcription profiles. Each of the graphs in the figure depicts schematically the change in transcription (vertical axis) from time t_0 (immediately previous to shift down) to time $t_{7.5}$ (7.5 h) of both strains (as indicated under the DD profile at the bottom of the figure). The black line corresponds to the wild-type strain (M145), and the dark gray line to the Δ*phoP* mutant (INB101). Each profile is named by a two-character designation, in which the first character refers to the expression changes in the wild type and the second to the change in the mutant strain. "A" means increase (Augmentation), and "D," decrease. The lack of a detected change of expression is indicated by "0." Lower case letters refer to a relative smaller change in one strain. The three H_r values that define each profile are indicated at the top of each panel (see Section 2 for details). The total number of genes classified into a profile are indicated in round brackets (first number in bold refers to quality 1 profiles, second number is the total).

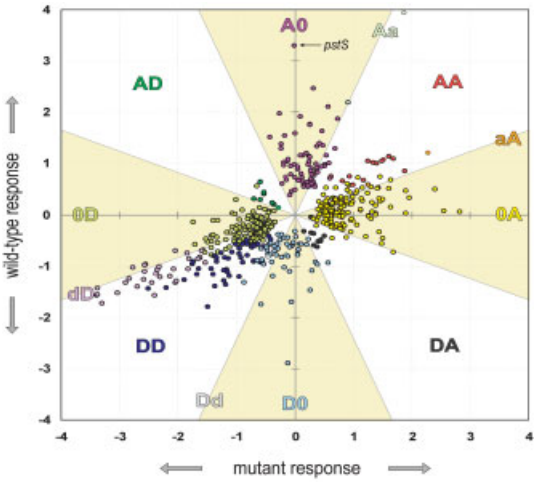

Figure 4. Vector analysis plot [41] showing the phosphate-limitation response of the statistically selected 529 genes that are differentially expressed. Vertical axis is the M_{c2} values and the horizontal axis is the M_{c4} values that define the wild type and mutant responses to phosphate limitation, respectively. The color of each circle corresponds to the color of the assigned profile, indicated in each sector.

3.3 Amino acids and protein synthesis genes

This group includes 62 genes related to translation that code for ribosomal proteins (47 genes), translation factors (5), aminoacyl-tRNA synthesis or modifying enzymes (5), proteins related to protein maturation (4), and rRNA modification (SCO1597) (Table 1A and Supporting Information). Of these genes, 50 are integrated in 11 clusters. These clusters also include genes involved in transcription (*rpoA*, *rpoB*, *rpoC*, *ssb*). Except in one case (*serS*), all these genes showed decreased transcription levels in the phosphate-depleted cultures, what is consistent with their role in protein synthesis. Indeed, the proteomic results detected the ribosomal protein S1, three translation factors [TU-1 (Fig. 6), Ts, ribosome recycling factor] and the Glu-tRNAGln amidotransferase subunit B with reduced synthesis after phosphate shift down (Table 2). Similar changes in transcription of this group of genes have been detected for the *C. glutamicum* and *E. coli* phosphate regulons [3, 19].

Differential transcription between strains was clearly detected. Most genes in this group showed profiles 0D or dD that indicate an upregulation of these genes mediated by PhoP (Table 1A; the zero in the 0D profile means lack of statistically significant change in the wild-type strain). Especially significant is the coincidence of profiles found in the 21 genes in the cluster *rpsJ* (SCO4701)-*rplO* (SCO4721). A *phoP*-dependent positive effect is also suggested by the profiles of amino acid transport (*gluABCD*, 0D profiles) and biosynthetic genes (*ask*, SCO4293, *sahH*, 0D profiles; *trpE*, A0; Table 1A). However, the *S*-adenosyl-L-homocysteine hydrolase protein (*sahH*) showed a different behavior (see Section 3.14).

On the other hand, a wild-type *vs.* mutant downregulation was observed for biosynthetic genes SCO0992 (putative cysteine synthase), *hisC2* and *aspC*, and for three genes encoding aminoacyl-tRNA synthetases (Table 1A).

3.4 Nucleotide metabolism genes

This group of genes includes seven genes for biosynthesis of nucleotides which showed strain-dependent expression (Table 1B). Three genes of the biosynthesis of purines, *purE* and the *purF–purM* operon, showed the 0A profile. On the other hand, the *purN* gene and the operon *purSQL* involved in the purine pathway (fourth step of the pathway), showed decreased transcription in both strains, but lower in the Δ*phoP* mutant strain (Table 1B).

Interestingly, the profile 0A of *pyrR*, the regulator of pyrimidine biosynthesis, can be correlated with the profile 0D of the structural *pyrH* gene. These profiles are in opposite positions in the diagram of Fig. 3. It is suggested that PhoP is responsible of a downregulation of *pyrR*, what in turn maintains the transcription of *pyrH*.

Table 1. Transcriptional responses to phosphate limitation[a)]

Systematic name	Gene name	Product	Profile	M_{c2}	M_{c4}	*p*-value contrast 2	*p*-value contrast 4	*p*-value contrast 5
(A) Amino acids metabolism, transcription, and translation								
Wild type *vs.* mutant upregulation								
SCO1597		rRNA methylase	**0D**	**−0.09**	**−0.59**	0.529	**<0.001**	0.013
SCO1598	*rplT*	Ribosomal protein L20	**dD**	**−0.85**	**−1.63**	**<0.001**	**<0.001**	0.009
SCO1599	*rpmI*	Ribosomal protein L35	**dD**	**−1.06**	**−2.08**	**<0.001**	**<0.001**	0.002
SCO2117	*trpE*	Anthranilate synthase	**A0**	**1.38**	**−0.23**	**0.001**	0.555	0.006
SCO2563	*rspT*[b)]	Ribosomal protein S20	**DD**	**−1.42**	**−2.19**	**<0.001**	**<0.001**	0.027
SCO3023	*sahH*	*S*-adenosyl-L-homocysteine hydrolase	**0D**	**0.43**	**−0.53**	0.047	0.016	0.003
SCO3615	*ask*	Aspartokinase	**0D**	**−0.25**	**−0.66**	0.084	**<0.001**	0.045
SCO3906	*rpsF*	Ribosomal protein S6	**dD**	**−1.41**	**−2.70**	**<0.001**	**<0.001**	**<0.001**
SCO3907	*ssb*[b,c)]	Single-strand DNA-binding protein	**DD**	**−0.81**	**−1.37**	**<0.001**	**<0.001**	0.055
SCO4293		Threonine synthase	**0D**	**−0.26**	**−0.97**	0.180	**<0.001**	0.015
SCO4652	*rplJ*	Ribosomal protein L10	**0D**	**−0.69**	**−1.58**	0.013	**<0.001**	0.025
SCO4653	*rplL*	Ribosomal protein L7/L12	**dD**	**−0.70**	**−2.05**	**<0.001**	**<0.001**	**<0.001**
SCO4654	*rpoB*	RNA polymerase beta chain	**0D**	**−0.35**	**−1.12**	0.026	**<0.001**	0.001
SCO4655	*rpoC*	RNA polymerase beta' chain	**0D**	**−0.35**	**−1.17**	0.145	**<0.001**	0.022
SCO4659	*rpsL*	Ribosomal protein S12	**dD**	**−1.04**	**−2.78**	**<0.001**	**<0.001**	**<0.001**
SCO4660	*rpsG*	Ribosomal protein S7	**dD**	**−0.96**	**−2.55**	**<0.001**	**<0.001**	**<0.001**
SCO4661	*fusA*	Elongation factor G	**dD**	**−1.20**	**−2.14**	**<0.001**	**<0.001**	0.001
SCO4701	*rpsJ*[b,c)]	Ribosomal protein S10	**DD**	**−1.44**	**−2.50**	**<0.001**	**<0.001**	0.020
SCO4702	*rplC*	Ribosomal protein L3	**dD**	**−1.29**	**−2.04**	**<0.001**	**<0.001**	0.026
SCO4703	*rplD*[c)]	Ribosomal protein L4	**0D**	**−0.43**	**−0.98**	0.056	**<0.001**	0.079
SCO4704	*rplW*	Ribosomal protein L23	**dD**	**−1.25**	**−2.83**	**<0.001**	**<0.001**	**<0.001**
SCO4705	*rplB*	Ribosomal protein L2	**dD**	**−1.33**	**−2.44**	**<0.001**	**<0.001**	**<0.001**
SCO4706	*rpsS*	Ribosomal protein S19	**dD**	**−1.80**	**−4.15**	**<0.001**	**<0.001**	**<0.001**
SCO4707	*rplV*	Ribosomal protein L22	**dD**	**−1.57**	**−3.39**	**<0.001**	**<0.001**	**<0.001**
SCO4708	*rpsC*	Ribosomal protein S3	**dD**	**−1.26**	**−2.60**	**<0.001**	**<0.001**	**<0.001**
SCO4709	*rplP*	Ribosomal protein L16	**dD**	**−1.33**	**−2.90**	**<0.001**	**<0.001**	**<0.001**
SCO4710	*rpmC*	Ribosomal protein L29	**dD**	**−1.73**	**−3.31**	**<0.001**	**<0.001**	**<0.001**
SCO4711	*rpsQ*	Ribosomal protein S17	**dD**	**−1.56**	**−3.38**	**<0.001**	**<0.001**	**<0.001**
SCO4712	*rplN*	Ribosomal protein L14	**dD**	**−1.53**	**−3.05**	**<0.001**	**<0.001**	**<0.001**
SCO4713	*rplX*	Ribosomal protein L24	**dD**	**−1.44**	**−3.39**	**<0.001**	**<0.001**	**<0.001**
SCO4714	*rplE*	Ribosomal protein L5	**dD**	**−0.98**	**−2.58**	**<0.001**	**<0.001**	**<0.001**
SCO4715	*rpsN*	Ribosomal protein S14	**dD**	**−1.22**	**−2.31**	**<0.001**	**<0.001**	**<0.001**
SCO4716	*rpsH*	Ribosomal protein S8	**dD**	**−1.11**	**−2.58**	**<0.001**	**<0.001**	**<0.001**
SCO4717	*rplF*	Ribosomal protein L6	**dD**	**−1.09**	**−2.36**	**<0.001**	**<0.001**	**<0.001**
SCO4718	*rplR*	Ribosomal protein L18	**dD**	**−1.06**	**−1.83**	**<0.001**	**<0.001**	0.007
SCO4719	*rpsE*	Ribosomal protein S5	**dD**	**−0.86**	**−1.81**	**<0.001**	**<0.001**	**<0.001**
SCO4720	*rpmD*	Ribosomal protein L30	**0D**	**−0.64**	**−1.44**	0.001	**<0.001**	0.005
SCO4721	*rplO*	Ribosomal protein L15	**0D**	**−0.74**	**−1.54**	0.002	**<0.001**	0.014
SCO4727	*rpsM*[b,c)]	Ribosomal protein S13	**DD**	**−0.92**	**−1.74**	**0.001**	**<0.001**	0.029
SCO4728	*rpsK*	Ribosomal protein S11	**dD**	**−0.84**	**−1.63**	**<0.001**	**<0.001**	0.002
SCO4729	*rpoA*[c)]	RNA polymerase alpha chain	**0D**	**−0.63**	**−1.13**	0.002	**<0.001**	0.064
SCO4730	*rplQ*	Ribosomal protein L17	**dD**	**−1.02**	**−2.32**	**<0.001**	**<0.001**	**<0.001**
SCO4734	*rplM*	Ribosomal protein L13	**dD**	**−0.87**	**−1.65**	**<0.001**	**<0.001**	0.007
SCO4735	*rpsI*[b,c)]	Ribosomal protein S9	**DD**	**−0.81**	**−1.36**	**<0.001**	**<0.001**	0.022
SCO5595	*rplS*	Ribosomal protein L19	**0D**	**−0.55**	**−1.32**	0.004	**<0.001**	0.005
SCO5774	*gluD*[c,d)]	Glutamate permease	**0D**	**−0.16**	**−0.64**	0.374	**0.001**	0.061
SCO5775	*gluC*	Glutamate permease	**0D**	**−0.26**	**−0.95**	0.120	**<0.001**	0.005
SCO5776	*gluB*	Glutamate-binding protein	**0D**	**−0.54**	**−1.49**	0.038	**<0.001**	0.011
SCO5777	*gluA*	Glutamate uptake system ATP-binding protein	**0D**	**−0.29**	**−1.00**	0.101	**<0.001**	0.007
Wild type *vs.* mutant downregulation								
SCO0992		Cysteine synthase	**DA**	**−0.52**	**0.44**	0.003	0.014	**<0.001**
SCO3961	*serS*	Seryl-tRNA synthase	**0A**	**−0.21**	**0.36**	0.063	**0.003**	0.001
SCO3792[c)]		Methionyl-tRNA synthetase	**D0**	**−0.50**	**−0.26**	**0.001**	0.059	0.204

Table 1. Continued

Systematic name	Gene name	Product	Profile	M_{c2}	M_{c4}	*p*-value contrast 2	*p*-value contrast 4	*p*-value contrast 5
SCO3795	*aspS*	Aspartyl-tRNA synthetase	**D0**	**−0.61**	**−0.06**	**0.001**	0.727	0.028
SCO3944	*hisC2*	Histidinol-phophate aminotransferase	**D0**	**−0.67**	**−0.29**	**<0.001**	0.034	0.043
SCO4645	*aspC*	Aspartate aminotransferase	**D0**	**−0.64**	**−0.09**	**<0.001**	0.544	0.013
SCO5571	*rpmF*	50S ribosomal protein L32	**D0**	**−0.54**	**−0.02**	**0.001**	0.885	0.021
(B) Nucleotide metabolism								
Wild type *vs.* mutant upregulation								
SCO4077	*purS*[b,e]	Phosphoribosylformylglycinamidine synthase	**DD**	**−0.49**	**−0.82**	**<0.001**	**<0.001**	0.066
SCO4078	*purQ*	Phosphoribosylformylglycinamidine synthase I	**dD**	**−0.76**	**−1.85**	**0.001**	**<0.001**	0.001
SCO4079	*purL*[d]	Phosphoribosylformylglycinamidine synthase II	**0D**	**−0.41**	**−0.66**	0.005	**<0.001**	0.199
SCO5626	*pyrH*[d]	Uridylate kinase	**0D**	**−0.20**	**−0.59**	0.154	**<0.001**	0.052
Wild type *vs.* mutant downregulation								
SCO1488	*pyrR*	Pyrimidine operon regulatory protein	**0A**	**−0.19**	**0.60**	0.244	**0.001**	0.001
SCO3059	*purE*	Phosphoribosylaminoimidazole carboxylase catalytic subunit PurE	**0A**	**0.15**	**0.57**	0.194	**<0.001**	0.012
SCO4086	*purF*	Amidophosphoribosyltransferase	**0A**	**0.32**	**1.13**	0.126	**<0.001**	0.008
SCO4087	*purM*[d]	Phosphoribosylformylglycinamidine cyclo-ligase	**0A**	**0.32**	**0.69**	0.124	**0.002**	0.210
(C) Respiration								
Wild type *vs.* mutant upregulation								
SCO0212		Hypothetical	**0D**	**0.03**	**−1.07**	0.921	**0.001**	0.012
SCO0213	*narK2*[f]	Nitrate/nitrite transporter	**0D**	**−0.04**	**−0.49**	0.766	**0.001**	0.020
SCO0216	*narG2*[f]	Nitrate reductase alpha chain	**0D**	**0.01**	**−1.03**	0.971	**<0.001**	0.007
SCO0217	*narH2*[c,d,f]	Nitrate reductase beta chain	**0D**	**0.00**	**−0.69**	0.990	0.009	0.055
SCO0922[c]		Succinate dehydrogenase iron–sulfur protein	**0D**	**−0.49**	**−0.74**	0.005	**<0.001**	0.299
SCO0923		Succinate dehydrogenase flavoprotein subunit	**dD**	**−1.15**	**−2.38**	**0.001**	**<0.001**	0.009
SCO0924		Cytochrome *b* subunit	**dD**	**−0.94**	**−1.53**	**<0.001**	**<0.001**	0.029
SCO4947	*narG3*[f]	Nitrate reductase alpha chain	**A0**	**1.33**	**0.38**	**<0.001**	0.176	0.018
SCO4950	*narI3*[f]	Nitrate reductase gamma chain	**A0**	**1.15**	**0.23**	**<0.001**	0.293	0.005
SCO6534	*narH*[f]	Nitrate reductase beta chain	**A0**	**0.47**	**−0.07**	0.008	0.694	0.029
SCO6535	*narG*[f]	Nitrate reductase alpha chain	**AD**	**0.22**	**−0.39**	0.165	0.021	0.01
Wild type *vs.* mutant downregulation								
SCO2149	*qcrA*	Rieske iron–sulfur protein	**D0**	**−0.57**	**0.08**	**0.001**	0.582	0.004
SCO2150	*qcrC*	Cytochrome *c* heme-binding subunit	**D0**	**−0.68**	**0.07**	**<0.001**	0.562	**<0.001**
SCO2155	*cox1*	Cytochrome *c* oxidase subunit I	**D0**	**−0.57**	**0.11**	**<0.001**	0.414	0.001
SCO3946	*cydB*	Cytochrome *bd* oxidase subunit II	**D0**	**−0.79**	**0.05**	**<0.001**	0.786	0.003
SCO3965		Conserved	**D0**	**−1.70**	**−0.05**	**<0.001**	0.811	**<0.001**
SCO3966[e]		Cytochrome *c* oxidase assembly factor	**D0**	**−1.03**	**−0.18**	**<0.001**	0.320	0.002
SCO3967		Conserved	**D0**	**−1.47**	**0.27**	**<0.001**	0.105	**<0.001**
SCO3968		Membrane protein	**D0**	**−0.93**	**0.36**	**<0.001**	0.073	**<0.001**
(D) Cell envelop biosynthesis								
Wild type *vs.* mutant upregulation								
SCO4873[e]		Possible *N*-acetylmannosamine-6-phosphate 2-epimerase	**A0**	**1.55**	**−0.18**	**<0.001**	0.141	**<0.001**
SCO4875[e]		Bactoprenol glucosyltransferase	**A0**	**1.16**	**0.09**	**<0.001**	0.698	0.003
SCO4879		Conserved	**A0**	**0.69**	**−0.15**	**<0.001**	0.343	**0.001**
SCO4880	*neuA*[e]	CMP-*N*-acetylneuraminic acid synthetase	**A0**	**0.79**	**0.28**	**<0.001**	0.062	0.017
SCO4881	*neuB*[e]	*N*-acetylneuraminic acid synthase	**A0**	**1.03**	**−0.19**	**0.003**	0.565	0.013
(E) Oxidative stress and iron metabolism								
Wild type *vs.* mutant upregulation								
SCO0379	*catA*	Catalase	**A0**	**1.16**	**0.14**	**<0.001**	0.414	**<0.001**
SCO0380		Hypothetical	**A0**	**1.00**	**0.36**	**<0.001**	0.066	0.022
SCO0560	*cpeB*	Catalase/peroxidase	**A0**	**0.99**	**0.22**	**<0.001**	0.208	0.003
SCO2113	*bfr*	Bacterioferritin	**A0**	**1.51**	**−0.43**	**0.001**	0.313	0.003

Table 1. Continued

Systematic name	Gene name	Product	Profile	M_{c2}	M_{c4}	*p*-value contrast 2	*p*-value contrast 4	*p*-value contrast 5
SCO2114[e]		Bacterioferritin-associated ferredoxin	**A0**	**1.30**	**−0.24**	**0.001**	0.533	0.007
Wild type *vs.* mutant downregulation								
SCO0494	*cchF*	Iron-siderophore binding lipoprotein	**D0**	**−0.82**	**−0.12**	**<0.001**	0.533	0.011
SCO0495	*cchE*[d]	Iron-siderophore ABC-transporter ATPase	**D0**	**−0.46**	**−0.17**	**<0.001**	0.143	0.082
SCO0498	*cchB*	Peptide monooxygenase	**D0**	**−0.57**	**−0.13**	**<0.001**	0.365	0.032
SCO0885	*trxC*[c]	Thioredoxin	**0A**	**0.09**	**0.49**	0.598	0.009	0.115
SCO5032	*ahpC*	Alkyl hydroperoxide reductase	**0A**	**0.04**	**0.68**	0.821	**0.002**	0.029
SCO5217	*rsrA*	Anti-sigma factor	**0A**	**−0.01**	**0.69**	0.975	**<0.001**	0.009
SCO5254	*sodN*	Superoxide dismutase	**aA**	**1.20**	**2.28**	**<0.001**	**<0.001**	0.012
(F) Nitrogen metabolism								
Wild type *vs.* mutant upregulation								
SCO2197		Membrane protein	**0A**	**0.22**	**0.88**	0.232	**<0.001**	0.017
SCO2198	*glnA*[c,d]	Glutamine synthetase	**0A**	**−0.08**	**0.32**	0.693	0.112	0.158
SCO2199[d]		Membrane protein	**0A**	**0.25**	**0.65**	0.129	**<0.001**	0.085
SCO2210	*glnII*	Glutamine synthetase	**0A**	**0.06**	**2.82**	0.806	**<0.001**	**<0.001**
SCO5583	*amtB*	Ammonium transporter	**0A**	**0.01**	**1.38**	0.971	**<0.001**	**<0.001**
SCO5584	*glnK*	Regulator	**0A**	**0.08**	**2.54**	0.749	**<0.001**	**<0.001**
(G) Phosphate metabolism								
Wild type *vs.* mutant upregulation								
SCO1565		Glycerophosphoryl diester phosphodiesterase	**A0**	**1.05**	**−0.14**	**0.009**	0.727	0.036
SCO1968		Glycerophosphoryl diester phosphodiesterase	**A0**	**1.98**	**−0.27**	**<0.001**	0.158	**<0.001**
SCO4139	*pstB*	Phosphate transporter, ATPase component	**A0**	**1.21**	**0.19**	**<0.001**	0.215	**<0.001**
SCO4140	*pstA*	Phosphate transporter, permease component	**A0**	**1.08**	**−0.11**	**<0.001**	0.643	0.001
SCO4141	*pstC*	Phosphate transporter, permease component	**A0**	**1.75**	**0.26**	**<0.001**	0.057	**<0.001**
SCO4142	*pstS*	Phosphate transporter, periplasmic component	**A0**	**3.31**	**−0.01**	**<0.001**	0.959	**<0.001**
SCO4228	*phoU*[d]	PhoU regulator	**A0**	**0.70**	**0.27**	**0.001**	0.186	0.143
SCO4229	*phoR*	PhoR sensor kinase	**A0**	**1.59**	**0.00**	**<0.001**	0.984	**<0.001**
SCO4230	*phoP*[c,d]	PhoP response regulator	**A0**	**0.57**	**0.22**	**<0.001**	0.143	0.106
SCO7697		Phytase	**A0**	**0.73**	**0.00**	**<0.001**	0.979	0.001
Wild type *vs.* mutant downregulation								
SCO2532	*phoH*	PhoH-like protein	**0A**	**−0.19**	**1.06**	0.54	**0.002**	0.008
SCO2533		Conserved	**0A**	**−0.23**	**0.76**	0.292	**0.002**	0.003
(H) Morphological differentiation								
Wild type *vs.* mutant upregulation								
SCO2954	*sigU*[g]	Transcriptional regulator	**Aa**	**2.20**	**0.91**	**<0.001**	**0.001**	**0.001**
Wild type *vs.* mutant downregulation								
SCO1674	*chpC*	Chaplin C	**D0**	**−0.83**	**−0.22**	**<0.001**	0.198	0.016
SCO3579	*wblA*	Transcriptional regulator	**0A**	**0.74**	**1.45**	0.002	**<0.001**	0.030
SCO5189		Hypothetical	**0A**	**−0.72**	**1.68**	0.006	**<0.001**	**<0.001**
SCO5190	*wblC*	Transcriptional regulator	**0A**	**−0.33**	**1.45**	0.067	**<0.001**	**<0.001**
SCO5191		Hypothetical	**0A**	**−0.03**	**1.26**	0.855	**<0.001**	**<0.001**
SCO7251		Hypothetical	**0A**	**0.09**	**1.13**	0.662	**<0.001**	0.001
SCO7252		Transcriptional regulator	**0A**	**0.33**	**1.96**	0.13	**<0.001**	**<0.001**
(I) Secondary metabolites								
Wild type *vs.* mutant downregulation								
SCO5877	*redD*	Transcriptional regulator	**0A**	**0.43**	**1.24**	0.006	**<0.001**	**0.001**
SCO5878	*redX*	Polyketide synthase	**0A**	**0.17**	**0.84**	0.372	**<0.001**	0.020
SCO5880	*redY*	RedY protein	**0A**	**0.47**	**1.89**	0.021	**<0.001**	**<0.001**
SCO5887	*redQ*	Acyl carrier protein	**0A**	**0.49**	**2.39**	0.087	**<0.001**	**<0.001**
SCO5888	*redP*	3-Oxoacyl-[acyl-carrier-protein] synthase	**0A**	**0.30**	**1.50**	0.223	**<0.001**	0.001
SCO5890	*redN*	8-Amino-7-oxononanoate synthase	**0A**	**0.51**	**1.60**	0.042	**<0.001**	0.003

Table 1. Continued

Systematic name	Gene name	Product	Profile	M_{c2}	M_{c4}	*p*-value contrast 2	*p*-value contrast 4	*p*-value contrast 5
SCO5891	*redM*	Peptide synthase	**0A**	**−0.08**	**0.98**	0.683	**<0.001**	0.001
SCO5893	*redK*	Oxidoreductase	**0A**	**0.13**	**0.78**	0.448	**<0.001**	0.011
SCO5896	*redH*	Phosphoenolpyruvate-utilizing enzyme	**0A**	**0.54**	**1.31**	0.006	**<0.001**	0.006
SCO5897	*redG*[c,d)]	Oxidase	**0A**	**0.52**	**1.41**	0.166	**0.001**	0.095
SCO6273	*cpkA*[h)]	Polyketide synthase	**0A**	**−0.02**	**0.52**	0.895	**0.002**	0.016
SCO6282	*cpkI*[h)]	3-Oxoacyl-[acyl-carrier protein] reductase	**D0**	**−2.90**	**−0.12**	**<0.001**	0.718	**<0.001**
SCO6283	*cpkJ*[h)]	Nucleoside-diphosphate-sugar epimerase	**D0**	**−1.10**	**−0.09**	**<0.001**	0.642	**<0.001**
SCO6284	*cpkK*[h)]	Acyl-CoA carboxylase, beta-subunit	**D0**	**−0.97**	**0.40**	**<0.001**	0.064	**<0.001**

a) Transcription profile type, M_c and *p*-values are shown for 140 selected genes. The selection criteria were: (1) PhoP-dependent transcription (excluded profiles AA and DD), 2) *p*-value < 0.05 of contrast 5. Some genes that did not meet both criteria are also included (see footnotes[b, c,] and [d]). Genes are ordered, firstly by functional class, secondly by the type of PhoP regulation, and then by chromosomal position with the aim of highlighting the coincidence of profiles among clustered genes. The primary annotation source is the ScoDB server (www.streptomyces.org.uk). For simplicity, designations "putative" have been removed. The *p*-values for contrasts 2, 4, and 5 are indicated in bold type when found significant after adjustment for multiple testing ($p<0.05$; see Section 2).
b) Despite being classified as profile DD, this gene is included since its M_c values reflect the dD profile of genes functionally related.
c) Despite a *p*-value >0.05 of contrast 5, the "nestedF" test is significant for this contrast.
d) Gene include because the profile shown match those of genes functionally related.
e) According to our own results of homology and motif searches.
f) According to ref. [48].
g) According to ref. [65].
h) According to ref. [68].

3.5 Respiration genes

Nineteen genes involved in oxidative phosphorylation were found to be differentially regulated between strains (Table 1C). Firstly, profile D0 was assigned for three genes (*qcrA*, *qcrC*, and *cox1*) coding for components of the cytochrome terminal oxidases, for the gene *cydB*, coding for the cytochrome *bd* oxidase subunit II, and for the genes SCO3965–SCO3968. The protein encoded by SCO3966 is a putative cytochrome *c* oxidase assembly factor, and a related function is assumed for the other gene products of the cluster (conserved and membrane proteins), because they share the same transcription pattern. Secondly, a possible PhoP-dependent positive effect (profiles 0D, dD, A0, and AD) was found for respiratory genes SCO0922, SCO0923 (succinate dehydrogenase subunits), and SCO0924 (cytochrome *b* subunit), and for components of nitrate reductase (Table 1C). There are three nitrate reductase clusters in *S. coelicolor*, each one including the structural genes *narG*, *narH* and *narI*, and the chaperone *narJ* [48]. In contrast to those genes, no significant differences between strains were found for six genes of the F_0F_1-ATP synthetase operon (*atpB*, *atpF*, *atpH*, *atpA*, *atpG*, and *atpD*) [49, 50], which showed a similar decreased transcription after phosphate shift down in both strains (Supporting Information). Indeed, a DD profile was found for the AtpA protein by 2-D analysis (Table 2).

Taken together all these results indicate that phosphate starvation produces a PhoP-dependent switch in energy metabolism, downregulating the aerobic respiration genes (terminal oxidases), and upregulating the nitrate reductase clusters (see Section 4).

3.6 Glycolysis and central metabolism

The only detected significant differences between the wild type and Δ*phoP* strain responses are those of the genes *gabD* (succinate-semialdehyde dehydrogenase, profile 0A), and SCO7040, one of the three paralogs coding for glyceraldehyde-3-phosphate dehydrogenases (profile 0D).

The gene SCO7630, coding for a putative phosphoglycerate mutase, shows a biologically significant A0 profile. In addition, the paralog phosphoglycerate mutase (SCO4209), which is not present in the used microarray slides, was also detected in 2-D gels under two protein variants showing the profiles AA and A0, respectively (Table 2). Both results suggest a PhoP-dependent activation of this activity to compensate for substrate limitation. Since this enzyme catalyzes the first reaction that needs inorganic phosphate as substrate, it has been suggested that this is a bottleneck for the glycolisis pathway under phosphate limitation [51]. Therefore, PhoP-mediated activation of this phosphoglycerate mutase gene could compensate for substrate limitation.

3.7 Genes involved in cell envelope biosynthesis

In the Gram-positive *B. subtilis*, PhoP regulates the cell wall secondary polymer replacement after phosphate limitation. PhoP represses the genes for teichoic acid synthesis that

Table 2. Protein changes in the proteome of the wild type and Δ*phoP*-lacking mutant of *S. coelicolor* under phosphate-limitation conditions

Spot[a)]	Systematic name	Gene name	Product	2-D profile	Variants[b)]
(A)					
1	SCO1773	–	Putative L-alanine dehydrogenase	AA	+
2	SCO4209	*pgm*	Phosphoglycerate mutase	AA/A0	+
3	SCO1947	*gap1*	Glyceraldehyde-3-phosphate dehydrogenase	AA	–
4	SCO4662	*tuf1*	Elongation factor TU-1	DD/(A0)	+
5	SCO5625	*tsf*	Elongation factor Ts	DD	–
6	SCO5627	*frr*	Ribosome recycling factor	DD	–
7	SCO1998	*rpsA*	30S ribosomal protein S1	DD	–
8	SCO4761	*groES*	Cochaperonin GroES	DD	–
9	SCO3671	*dnaK*	DnaK, heat-shock protein 70	DD	–
10	SCO5371	*atpA*	ATP synthase alpha chain	DD	–
11	SCO5501	*gatB*	Probable Glu-tRNA-Gln amidotransferase subunit B	DD	–
12	SCO3767	–	Hypothetical protein	DD	–
13	SCO4293	–	Putative threonine synthase	DD	–
14	SCO4230	*phoP*	Putative response regulator	A0	–
15	SCO2198	*glnA*	Glutamine synthetase I	0A	–
16	SCO3023	*sahH*	*S*-adenosyl-L-homocysteine hydrolase	0A	–
17	SCO6282	–	Putative 3-oxoacyl-[acyl-carrier protein] reductase	D0	–
18	SCO3899	–	Inositol-1-phosphate synthase	D0	–
(B)					
19	SCO2180	*pdhL*	Putative dihydrolipoamide dehydrogenase	AA	–
20	SCO7000	*idh*	Isocitrate dehydrogenase	AA	–
21	SCO4827	*mdh*	Malate dehydrogenase	AA	–
22	SCO3622	–	Putative aminotransferase	AA	–
23	SCO5743	–	Thymidylate synthase	AA	–
24	SCO0596	*dpsA*	DNA-binding protein	DD	–
25	SCO2901	–	Hypothetical protein	DD	–
26	SCO4411	–	Calcium binding protein	DD	–
27	SCO4771	–	Putative inosine-5′-monophosphate dehydrogenase	A0	–
28	SCO4809	*sucD*	Succinyl-CoA synthetase alpha chain	A0	+
29	SCO4366	–	Putative phosphoserine aminotransferase	0A	–
30	SCO4723	*adk*	Adenylate kinase	0A	–
31	SCO0179	–	Putative zinc-containing dehydrogenase	D0/00	+
32	SCO2158	–	Putative kinase	0D	–
33	SCO2440	–	Putative transcriptional regulator	0D	–
34	SCO4277	–	Putative tellurium resistance protein	0D	+
35	SCO4636	–	Hypothetical protein	0D	–

(A) Proteins which encoding genes belong to the group of 529 genes selected from the transcriptomic results. The proteins encoded by the *pgm*, *frr*, *rpsA*, *dnaK*, and *gatB* genes are also included because of these genes are clustered or are functionally related with other selected genes (see text for details).

(B) Proteins which encoding genes do not have statistically significant transcriptomic results.

a) Spot numbers are indicated in concordance with Fig. 6.

b) Detected protein variants are indicated.

forms the phosphate-rich secondary polymer, and activates the biosynthetic genes for the phosphorous-free teichuronic acid, as a way to obtain phosphate [52]. Teichoic acids as a phosphorous storage have also been reported in *Streptomyces* [53]. The biosynthetic genes for both cell wall polymers have not yet been identified in the *S. coelicolor* genome. However, we have identified five clustered genes with putative roles on the biosynthesis of cell wall components that showed the A0 profile (Table 1D), and includes the putative genes *neuA* and *neuB*, encoding enzymes involved in *N*-acetyl neuraminic acid biosynthesis. A putative PhoP-binding site (14.28 bits) has been located in the bidirectional promoter region SCO4873-SCO4874; and another PhoP-binding site has experimentally validated in the promoter region of SCO4879 (see Section 3.16).

3.8 Stress response genes

Universal stress proteins (Usp) have been found across genomes of Archaea, Bacteria, and Eukarya. Its synthesis is induced in response to a large number of stresses [54], including phosphate starvation. We have identified four *S. coelicolor* genes containing the PF00582 motif characteristic of the *E. coli* Usp proteins. These genes cluster in two close regions (SCO0180–SCO181 and SCO0198–SCO200; the last cluster including a zinc-dependent alcohol dehydrogenase). Their transcriptions decreased in phosphate limitation, but a greater transcription decrease was observed in the mutant strain.

A group of seven genes code for chaperones, heat-shock and cold-shock proteins. They are the *tig* gene (cell division trigger factor), chaperone *sugE*, chaperonins *groES* and *groEL1*, and cold-shock proteins *scoF1* and *scoF4*. All of them, except the chaperonin *groEL2*, showed decreased transcription in response to phosphate limitation without any significant differences between strains, as occurs in *Sinorhizobium meliloti* [20]. The same patterns were found for the DnaK and GroES proteins in the bidimensional electrophoresis, which are part of the *dnaK* operon (*dnaK*, *grpE*, *dnaJ*, *hspR*) and *groES-groEL1*, respectively [55].

3.9 Oxidative stress and iron metabolism genes

It is known that *E. coli* cells undergo oxidative stress after phosphate limitation and that the cells respond by increasing the catalase activity [56, 57]. Yuan *et al.* [58] have recently reported that the catalase gene is activated by phosphate limitation in *S. meliloti*, *Pseudomonas aeruginosa*, and *Agrobacterium tumefaciens*. In our results, the transcription of *catA*, for the major *S. coelicolor* vegetative catalase, and of a gene (SCO0380) located immediately downstream of *catA*, was activated under phosphate limitation only in the wild-type strain (profile A0). The same occurs with the catalase/peroxidase gene *cpeB* (Table 1E). However, bioinformatic searches in the *catA* promoter region of *S. coelicolor* did not reveal any putative PHO box sequence (see Section 3.16), suggesting that PhoP activates its transcription indirectly.

The two superoxide dismutases known in *S. coelicolor* [59] showed opposite responses to phosphate limitation. The transcription of the *sodF* gene, encoding the iron- and zinc-containing superoxide dismutase, decreased in both strains (DD profile), but the nickel-containing superoxide dismutase gene (*sodN*) was overexpressed and higher in the mutant strain (aA profile; Table 1E). In addition, the alkyl hydroperoxide reductase *ahpC* gene transcription was higher in the mutant strain (0A profile).

The bacterioferritin gene *bfr* and the downstream gene SCO2114 (bacterioferritin-associated ferredoxin) had the A0 profile, indicating that their expression is PhoP dependent, as in *S. meliloti* [20]. It has been suggested that the primary function of bacterioferritins is the detoxification of iron and protection against radical oxygen species [60]. Other iron-related genes are those of the coelichelin biosynthesis cluster genes *cchB* (peptide monooxygenase), *cchE* (iron-siderophore ABC-transporter ATPase), and *cchF* (iron-siderophore binding lipoprotein); their transcription decreases most in the wild type at t_0 (profiles D0). Although iron is an essential nutrient, it is also problematic due to its ability to catalyze Fenton reactions that generate reactive oxygen species. These data suggest that the PHO response includes the prevention of this source of oxidative damage.

3.10 Nitrogen metabolism genes

Several genes involved in nitrogen metabolism (Table 1F) showed a characteristic overtranscription in the Δ*phoP* mutant (0A profile), suggesting that they are modulated by PhoP. This group includes *glnA* (glutamine synthetase I, beta subtype), that is flanked by two genes encoding membrane proteins showing the same profile; *glnII* (glutamine synthetase II), *amtB* (ammonium transporter), and *glnK* (PII protein). The GlnA protein has been identified in the bidimensional gels and the same pattern was revealed (Fig. 6, enlarged). The *amtB* and *glnK* genes form part of the *amtB–glnK–glnD* operon [61–63], where *glnD* is another post-translational regulator (PII nucleotidyltransferase).

3.11 Phosphate metabolism-related genes

Several *S. coelicolor* genes are involved in phosphate scavenging from organic phosphate compounds in soil, phosphate transport, and regulation (Table 1G). The operon *pstSCAB* encodes the phosphate-binding protein PstS and the components of the high affinity phosphate transport system. As expected – since the *pstS* promoter is PhoP activated [17, 45] – all of them showed the A0 profile. The *phoR–phoP* system is self-regulated [17]. Both these genes showed an A0 profile, indicating that they are PhoP regulated in agreement with the proteomic profile (Fig. 6). The *phoRP* cluster is transcribed from a bidirectional promoter region that also expresses *phoU* in the opposite orientation as a monocistronic transcript [16]. The profile of *phoU* was also A0, but the downstream genes, *mtpA* and SCO4226, showed clear AA profiles (Supporting Information). This confirms that the metallothionein *mtpA* gene – putatively involved in oxidative stress response – is upregulated when P_i was limiting by a PhoP-independent mechanism, as proposed recently in *S. lividans* [16].

Seven genes were found encoding glycerophosphodiester phosphodiesterases, whereas in *E. coli* there are only two: one periplasmic enzyme, GlpQ, and another cytosolic one, UgpQ. The genes SCO1565, SCO1968, and SCO7550 encode secreted glycerophosphodiesterases [64]. Four other genes encode cytoplasmic proteins (SCO1090, SCO1419, SCO3976, and SCO5661). Of these genes, SCO1565 and SCO1968 showed an A0 profile, and both genes are members of the PHO regulon since their promoters contain PhoP-binding sites (see Section 3.16).

The putative phytase encoded by SCO7697 (myo-inositol hexakisphosphate 3-phosphorylase) is also a phosphate-scavenging secreted enzyme. The gene showed the A0 profile, and is indeed regulated by PhoP, since it contains a PhoP-binding site in the promoter region (see Section 3.16). In *Bacillus licheniformis*, but not in *B. subtilis*, phytase accumulates extracellularly in conditions of phosphate starvation [22].

A different behavior (0A profile; Table 1G) was observed in the genes *phoH* (SCO2532), and SCO2533 (conserved protein) that belongs to a cluster of four genes. The function of PhoH, a known member of the PHO regulon in *E. coli*, is not clear. The 0A profile indicates that these genes could be repressed by PhoP. This repression must be indirect since no PHO box was found in their promoter regions (see Section 3.16).

3.12 Morphological differentiation

Eight genes related to morphological differentiation are listed in Table 1H. The extracytoplasmic sigma factor *sigU* that causes a delay in aerial mycelium formation when overexpressed [65], showed increased transcription; the response is higher in the wild type (profile Aa). The results of six other genes, putatively involved in sporulation, reflected 0A profiles. The hypothetical gene SCO7251 contains a TTA codon that is the target of *bldA* regulation. Finally, the chaplin (cell-surface proteins involved in aerial mycelium formation [66]) gene *chpC* decreased its expression mainly in the wild type (profile D0).

3.13 Secondary metabolites

S. coelicolor in postexponential cultures produces several chemical classes of antibiotics including extracellular actinorhodin and cell-bound prodigiosins. Prodigiosins are synthesized in MG medium, just after the end of the exponential phase, and actinorhodin production is delayed several hours (data not shown). Consequently, no expression was expected in the actinorhodin cluster genes at t_0 time (39 h), and no significant change was observed. On the other hand, ten of the genes belonging to the prodigiosins red cluster were expressed and showed 0A profiles (Table 1I). This profile (Fig. 5) indicates that transcription of these secondary metabolism genes is derepressed following phosphate deprivation and that this derepression is higher in the Δ*phoP* mutant.

The gene SCO7221 encoding a polyketide synthetase for germicidin biosynthesis [67] was expressed at the time used in the experiment showing an AA profile, although the transcription response was higher in the mutant strain (Supporting Information). Four genes of the cryptic polyketide synthetase cluster *cpk* [68] were found to be downregulated in a PhoP-dependent manner (Table 1I). The *cpkI* gene encodes a 3-oxoacyl-[acyl-carrier protein] reductase which has been clearly detected by the proteomics approach substantiating the D0 microarrays profile (see below; Fig. 6, enlarged area).

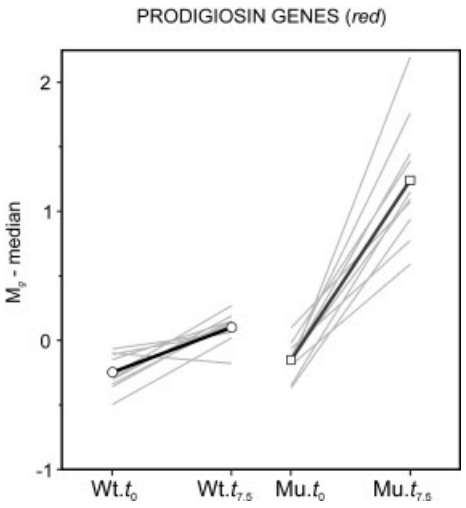

Figure 5. Transcription profiles of *red* genes. Ten prodigiosin biosynthesis genes, including the specific activator *redD*, showed significant changes. The vertical axis is the normalized M_g value. For each *red* gene, the mean of the four conditions M_g values was computed. Then, normalized M_g values were calculated as the subtraction. In this way, the coincidences between the transcription profiles are most evident. Gray lines are the plots of the transcription values in the wild-type strain (lines on the left), and in the mutant strain (on the right). The black line is the mean profile of the wild type, and the dark gray line corresponds to the mean profile of the mutant strain.

3.14 Proteomic analysis of phosphate deprivation

Proteomic studies to analyze the effect of phosphate deprivation on *S. coelicolor* M145 and in the *phoP*-lacking mutant *S. coelicolor* INB101 (Table 2) were made by using the same samples collected for RNA extraction. The *phoP*-gene deletion was confirmed by the proteomics studies, since no PhoP protein spot was observed in the Δ*phoP* mutant. Upregulation of the PhoP protein following phosphate limitation was only detected in the wild-type strain.

Changes in the levels of the first group of proteins (Table 2A) mostly agree with the microarray data and those have been explained in the microarrays section (see above). Therefore, only special behaviors will be explained in this section. Several proteins have been observed under two or more protein isoforms. The presence of protein variants has been described in different microorganisms; as an average, *E. coli* contains 1.18 variants *per* protein while *C. glutamicum* shows 1.15 variants, due to p*I* modifications or molecular mass variations [69]. Besides, in *S. coelicolor* 1.2 variants *per* protein have been reported, and several proteins involved in actinorhodin biosynthesis presented more than one form [21, 70]. A putative L-alanine dehydrogenase was detected in two different positions (spots) with similar molecular mass, but showing different p*I*. Both isoforms were induced by phosphate limitation and the most intensive induction effect was shown by the mutant strain (Fig. 6, enlarged). These

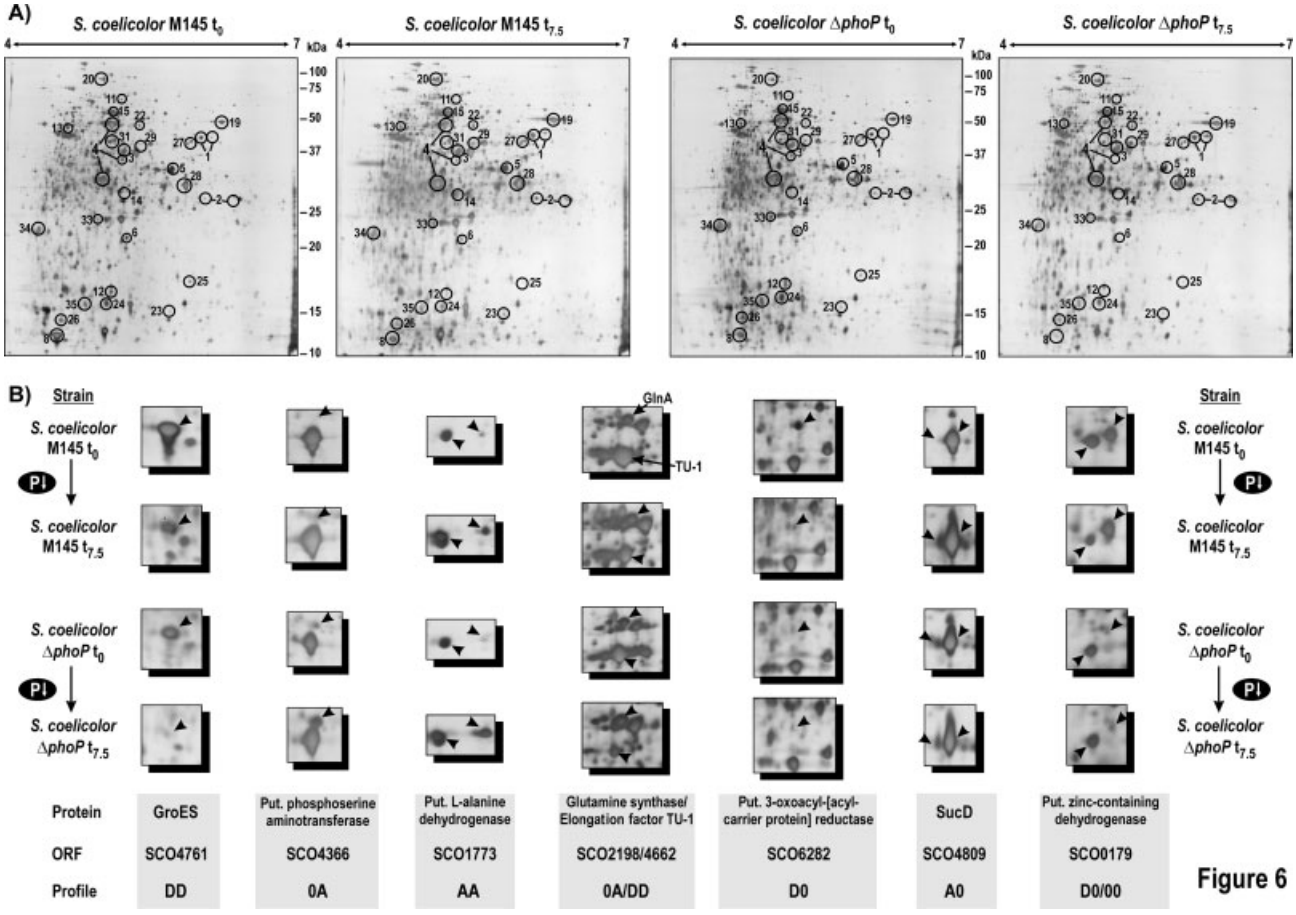

Figure 6. (A) Comparative 2-D gel analysis of phosphate-controlled cytoplasmic proteins of wild-type strain of *S. coelicolor* (M145) and the Δ*phoP* mutant (Δ*phoP*). Phosphate-replete (t_0) or phosphate-limited ($t_{7.5}$) conditions are shown. Encircled proteins correspond to those in Table 2. pH ranges (4.0–7.0) in first dimension are shown by arrows. (B) Some examples of protein profiles are enlarged. Protein spots are indicated by arrowheads. Protein name, gene identifier (ORF) and expression profile are shown in the lower part of the figure. Strain and phosphate conditions are specified (right and left). Put., putative protein.

variant forms suggest modifications in these proteins, probably affecting the activity of the protein, as proposed for SigH in *S. coelicolor* [71].

A tellurium resistance protein (SCO4277) was found as two protein variants with 0D profile (Table 2B). The tellurium resistance protein-encoding gene is not represented in the microarrays but one hypothetical protein (SCO3767, Table 2A) also related to tellurite resistance was found to be repressed under phosphate limitation. The biochemical function of tellurite resistance proteins is not clear but genes encoding these proteins are found in many pathogenic bacteria. The uptake of tellurite in *E. coli* occurs *via* P_i transport system [72]. It seems clear that tellurite-resistance proteins are regulated by phosphate.

The Glu-tRNAGln amidotransferase subunit B is downregulated following phosphate limitation (Table 2A). The encoding gene (*gatB*) is located in a gene cluster with the related genes *gatA* and *gatC*. This last gene showed a transcription profile DD in agreement with the observed shift down in proteome under phosphate deprivation in both strains.

The glyceraldehyde-3-phosphate dehydrogenase paralog named *gap1* showed discordance between proteomics and transcriptomics data (Table 2A), since that protein is upregulated under phosphate deficiency but the transcription pattern showed a D0 profile. A similar discordance was exhibited by *S*-adenosyl-L-homocysteine hydrolase. That differential gene and protein expression patterns has been frequently described [73, 74] and they appear to be due to posttranscriptional control mechanisms.

Some proteins that were not discussed in the microarrays section because their data were not statistically significant are included in Table 2B with their corresponding proteomic profiles. Three AA-profiled proteins: putative dihydrolipoamide dehydrogenase (SCO2180), isocitrate dehydrogenase (SCO7000), and malate dehydrogenase (SCO4827), directly involved in the TCA cycle have been detected by bidimensional gels. Moreover, a thymidylate

synthase (SCO5743) involved in the pyrimidine metabolism was induced under phosphate-limiting conditions reflecting the cellular response to obtain ATP from different monophosphate nucleotides.

A DD profile was shown by two proteins related with the oxidative damage protection: (i) a DNA-binding protein (SCO0596), which is a ferritin-like protein showing the presence of a "dps" motif (Pfam database [75]); these proteins bind to DNA without any apparent sequence specificity, to protect DNA from oxidative damage. (ii) A hypothetical protein (SCO2901) similar to members of the alkyl hydroperoxide reductase C/thiol-specific antioxidant family (Bcp, *M. tuberculosis* bacterioferritin comigratory proteins). Therefore, some proteins involved in oxidative stress protection show DD profiles indicating a PhoP-independent regulation, whereas other genes could be PhoP regulated (see Section 3.9).

Two calcium-binding protein paralogs have been found in *S. coelicolor* (SCO4411, SCO5464). SCO4411 was found by the proteome analysis to have a clear DD profile. These proteins are involved in signal transduction and their down-regulation could be related with the growth rate reduction.

Two other proteins listed in Table 2B were induced in the mutant strain under phosphate limitation. The first is a putative phosphoserine aminotransferase involved in vitamin B6 biosynthesis and in glycine, serine, and threonine metabolism pathways. Both pathways release phosphate providing it under phosphate-starvation conditions in the Δ*phoP* mutant. The other protein was adenylate kinase involved in purine metabolism that may be induced to provide energy (obtaining ATP).

The last group of proteins in Table 2B include proteins with a 0D profile. These include a putative kinase and a putative IclR-family transcriptional regulator.

3.15 Formation of some protein variants responds to PhoP control

Some protein variants exhibited distinct profiles, suggesting a differential regulation of expression, or PTMs. Thus, two phosphoglycerate mutase (SCO4209) variants showing the profiles AA and A0 were identified, while a paralog (SCO7630) phosphoglycerate mutase exhibited also an A0 profile (see Section 3.6). The existence of isoforms for the SCO4209 protein has been observed previously [21]. Another example of differences among protein variants was shown by the elongation factor TU-1, which was detected under three variant forms (Fig. 6, enlarged). Four variants of this protein have been found in *C. glutamicum* [69]. Interestingly, two TU-1 variants showed a DD profile (in concordance with the microarrays data), while one exhibited an A0 pattern.

Two other proteins in Table 2B showed the A0 profile. A putative inosine-5′-monophosphate dehydrogenase, which is involved in purine metabolism and the succinyl-CoA synthetase alpha chain (*sucD*), a component of the TCA cycle (identified under two protein variants). The major spot did not show variation in response to phosphate, while the acidic variant presents a clear A0 profile (Fig. 6, enlarged), *i.e.* succinyl-CoA synthetase modification responds to phosphate control. Isoforms of this protein have been reported previously [21].

A putative zinc-containing dehydrogenase (SCO0179) was identified under two proteins variants (Fig. 6, enlarged). First, a D0-profiled variant showing repression in the wild-type strain under phosphate-limited conditions. On the other hand, the second variant presents a special 00 profile, since it does not change by phosphate deprivation but it is poorly detected in the *phoP*-lacking strain when it is compared with the wild-type strain.

3.16 Validation of the PhoP-dependent transcriptional profiles: Identification of new PHO-regulon genes

We have previously described the target sequences of the regulator PhoP as composed of two or more DRus of 11 nucleotides [17]. Each two DRu constitute one PHO box. These DRus served to identify putative DRu in the promoter regions of the phytase gene SCO7697 (four DRu), and of glycerophosphodiesterase genes SCO1565 (three DRu) and SCO1968 (four DRu). The binding of PhoP to these promoter regions was demonstrated by EMSA (Fig. 7). These results validate the A0 transcriptional profiles of these genes and confirm that they are directly activated by PhoP.

To identify additional PhoP-binding sites, the sequences of 468 differentially transcribed genes, *i.e.* those which profiles were not AA or DD, and of seven genes which proteins were differentially synthesized (Table 2B), were scanned with a weight matrix (see Section 2). This search detected the known five PhoP sites and other 125 candidate sites (Supporting Information, Table S2). The sites found in the promoter regions of SCO0034 (containing three DRu; Ri = 21.37 bits), SCO2878 (five DRu; 18.57 bits), SCO4879 (three DRu; 13.23 bits), and SCO4261–SCO4262 (three DRu; 12.55 bits) were experimentally validated (Fig. 7). The highest affinity of PhoP was to the regulatory region of SCO4879 (Fig. 7). This gene encodes a conserved protein and is followed by the putative genes *neuA* and *neuB* (Table 1D), all of which showed A0 profiles. This suggests that the binding of PhoP to the SCO4879 regulatory region activates the transcription of the three clustered genes.

Transcription profiles of both SCO0034 and SCO2878 were of the type 0D which indicates a positive role for PhoP. Gene SCO0034 encodes a 159-amino acid protein that contains the helicase-associated domain, pfam03457, mainly found in *Streptomyces* proteins. Yeats *et al.* [76] have suggested a role for these proteins in the telomere formation of the linear chromosome.

The PHO box that controls two opposite genes, SCO4261 and SCO4262, separated by only 16 nucleotides, has a novel regulatory role and an unprecedented location. This PHO box is located in the coding region of SCO4262 (positions 26–

58). Both genes showed 0A profiles, what indicates a direct repressor function for PhoP. The SCO4261 gene encodes a response regulator of unknown function and SCO4262 encodes a hypothetical protein of 115 amino acids, and contains a TTA codon, target of *bldA* regulation.

We have also investigated the promoter region of the *phoH* gene, SCO2532 (see Section 3.11) for the presence of an undetected PhoP-binding site, since this gene is upregulated in the mutant strain (profile 0A). The electrophoretic mobility was unchanged (Fig. 7). It is possible that PhoP represses the transcription of *phoH via* an intermediate regulatory protein.

4 Discussion

In the soil-dwelling *Streptomyces* species, the concentration of inorganic phosphate in the culture media is one of the most important nutritional factors affecting both growth and differentiation [77], and secondary metabolite biosynthesis [6, 78]. Taking together the transcriptomic and proteomic results, we have observed that 551 genes of *S. coelicolor* had increased or decreased expression in at least one comparison between the experimental conditions (Fig. 2). Several factors account for the observed changes. Main factors are the growth arrest of the shift-down culture and the phosphate limitation that eventually triggers the PHO regulon. In addition, other media components varied in the half-spent medium at t_0 as compared to the fresh one at $t_{7.5}$. No major stress caused by manipulation of the cells during the shift down was observed, since the immediate growth after the shift down is exponential (Fig. S1). Moreover, general-stress genes are downregulated at $t_{7.5}$, thus excluding a stress-mediated induction of other genes. In order to discriminate these factors from the changes due to PhoP-dependent regulation, the responses of wild type and Δ*phoP* strains were compared following culture conditions that allowed a similar growth pattern. The statistical analysis lead us to distinguish eight major types of profiles. Four other intermediate profiles were also observed that allowed a fine-tuning in the classification of the responses (Figs. 3 and 4). We have focused on the analysis of genes with known biological function or with a plausible putative function. Data for the 368 genes not commented here are provided in the Supporting Information, Table S1. These set of genes also include 38 regulators, of unknown function at present, which are differentially transcribed in both strains.

The expression of a significant number of genes was the same in the wild type and in the Δ*phoP* mutant (profiles DD or AA). Therefore, these genes are not dependent upon PhoP and their expression is regulated by other factors. Alternatively, it is possible that other regulators of phosphate control can act as well in the shift-down cultures as suggested for the regulation of the *mtpA* gene in the close species *S. lividans* [16].

The results of this work confirmed that phosphate deprivation induces expression of the *phoR–phoP* cluster (A0 profile). The response regulator PhoP becomes phosphorylated following phosphate deprivation and the increased expression of PhoP allows the induction of a number of genes – the PHO regulon – by binding to the PHO boxes occurring in the promoters of these genes [17]. Matin *et al.* [46] have proposed that a two-step starvation process occurs in *E. coli*; the first step involves induction of enzymes to capture the scarce available phosphate. As shown in Table 1G, phosphate deprivation triggers a series of extracellular enzymes involved in phosphate scavenging from organic phosphates. These include a phytase gene and two glycerophosphodiester phosphodiesterase genes. These extracellular phosphodiesterases correspond functionally to the GlpQ of *E. coli*, a periplasmic enzyme that hydrolyzes glycerophosphodiesters into sn-glycerol-3-phosphate and the corresponding alcohol or amine. The PhoP-dependent induction of phytase is consistent with the fact that phytate is the major storage form of phosphate in some plant materials that eventually become part of the organic matter of the soil. The presence of PhoP-binding sites in these genes, validated by EMSA (Fig. 7), supports the conclusion that they belong to the PHO regulon.

One of the key proteins in phosphate scavenging is PstS, the extracellular phosphate-binding protein. The *pstS* promoter activity increased from basal levels up to 240-fold in the wild-type phosphate-limited cultures (Fig. 4). Indeed, PstS has also been found to be the most abundant protein induced in *B. subtilis* after phosphate starvation [4]. We

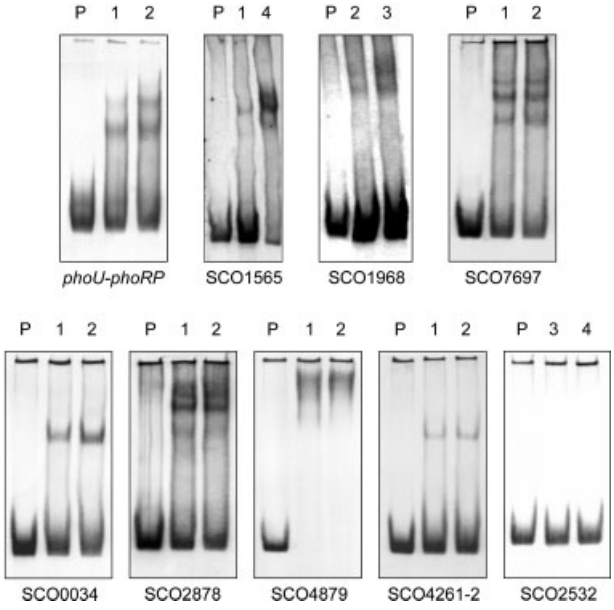

Figure 7. Electrophoretic mobility analysis (EMSA) of PhoP binding to different promoter regions. Lane P, probe without protein; Lane 1, 3.125 pmol of GST-PhoP^{DBD} protein; Lane 2, 6.25 pmol; Lane 3, 12.5 pmol; Lane 4, 25 pmol. The *phoU–phoRP* bidirectional promoter region was used as positive control.

reported previously that the *pstS* promoter is activated by PhoP binding in *S. coelicolor* [17]. This earlier finding validates the PhoP-dependent *pstS* profile A0 (Table 1G). Interestingly, PstS is induced by fructose and other sugars [45], indicating that an excess of these sugars triggers a phosphate response because of the high phosphate demand for sugar transport and phosphorylation deprives the cell of phosphate. The entire *pstSCAB* cluster, as expected for the components of the ABC-type phosphate transport system, is PhoP dependent, and probably is transcribed as an operon. This is also the case of the homologous genes of *E. coli*, *B. subtilis*, *S. meliloti*, and *Clostridium acetobutylicum*.

As a parallel strategy to survive hunger, *Streptomyces* cells may consume phosphorous storage material, such as polyphosphates, nucleotides, or teichoic acids [53, 79]. In *B. subtilis* phosphate-limited cells, teichoic acids are partially replaced by teichuronic acids, *via* the transcriptional repression/activation of the biosynthesis genes mediated by PhoP [52]. Two PhoP-binding sites, one of them validated by EMSA (Fig. 7), have been found in a cluster of genes putatively involved in the biosynthesis of cell wall polysaccharides. This activation indicates that also a replacement of phosphate-rich polymers with phosphate-free polymers could take place in the *Streptomyces* cell walls.

Analysis of the data obtained revealed interesting conclusions on other genes not obviously involved in phosphate metabolism. Most of the primary metabolism genes, particularly those involved in transcription, protein synthesis, cell division, and amino acid metabolism, were underexpressed in both the wild type and the Δ*phoP* mutant following phosphate deprivation. The growth arrest of the shift-down cultures accounts for the decreased expression. Remarkably, 58 of these genes showed a dD profile, *i.e.* their expression is upregulated in wild-type strain (Fig. 4). This regulation is also observed in the data of *S. meliloti*, although it was not discussed by the authors [20].

The cross regulation between phosphate deprivation and nitrogen metabolism is another interesting finding. *S. coelicolor* has an unusually large arsenal of glutamine synthases including a prokaryotic GS-beta subtype enzyme encoded by *glnA*, a eukaryotic-like glutamine synthetase II (encoded by *glnII*), and three annotated *glnA*-like genes [61]. The microarrays results of *glnA* regulation were validated by the proteomics findings (Fig. 6). The *amt* and *glnK* genes are part of the *amtB–glnK–glnD* operon [61], where *glnD* encodes a posttranslational regulator of the glutamine synthetase activity. The clustering of these genes suggests that their promoter might be phosphate regulated. The finding that *glnII* is also regulated by phosphate suggests that all those genes that are *glnR* and *glnRII* dependent [62, 63] are controlled by phosphate through a PhoP-mediated control of the *glnR* genes.

Eight genes of oxidative phosphorylation showed a D0 profile. Some of them encode subunits of terminal oxidases, whereas others are probably participating in this function (Table 1C). Related to this, the transcription response of the three nitrate reductase clusters is higher in the wild type than in the mutant strain. Transcription of genes of the second cluster remains constant in the wild type after the shift down (0D profile), whereas genes of the first and third clusters are upregulated in a PhoP-dependent manner (A0 or AD profiles; Table 1C). Taken together, those profiles indicate that phosphate-limiting conditions triggering the PhoP-mediated response result in a switch from aerobic to anaerobic respiration. Similarly, in *B. subtilis* several studies have revealed a regulatory loop between the PHO response and the cellular respiratory conditions [80].

The bioinformatics searches coupled to EMSA validation have revealed novel PhoP-binding sites in the promoter regions of SCO0034, SCO2878, and SCO4261–SCO4262. A putative role in the chromosome maintenance has been attributed to SCO0034, whereas SCO4261 is a putative response regulator. Although we do not know which genes depend on this regulator, it is an example of PhoP acting as a regulator of other regulators, what can explain expression responses of genes lacking PHO boxes in their promoter regions. An indirect cascade mechanism has also been suggested for the PhoP regulation of the *ppk* gene in *S. lividans* [79]. The complexity of the phosphate-limitation response is enriched by the observation of protein variants differently synthesized (Table 2).

This work provides also information on genes related to morphological differentiation and secondary metabolism. The transcriptomic data suggest a PhoP-dependent repression of genes related to differentiation at the time of culture collection ($t_{7.5}$). Most *Streptomyces* species do not sporulate in liquid culture, although the genetic program can be initiated [81]. All of the prodigiosin biosynthesis genes showed a 0A profile, indicating that they are derepressed in the Δ*phoP* strain (Fig. 5). These results correlate with the observation that the Δ*phoP* mutant grown on solid media showed increased pigment production and precocious sporulation, as observed previously in *S. lividans* [15]. Genes of cryptic cluster *cpk* were also derepressed in the Δ*phoP* strain (Table 1I). However, there is no evidence for direct binding of PhoP to the promoter of antibiotic biosynthesis genes [17, 82] indicating that the negative regulation of secondary metabolism genes is exerted by a PhoP-mediated signal transduction cascade that modulates pleiotropic regulatory genes that in turn activate pathway-specific regulators, rather than by direct binding of PhoP to the promoters of antibiotic biosynthesis genes. The alternative possibility that PhoP binds directly to the promoters of pathway-specific activators cannot be excluded at this time, but experimental support has not been found to substantiate this last hypothesis.

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