Cell envelope gene expression in phosphate-limited *Bacillus subtilis* cells

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The high phosphate content of *Bacillus subtilis* cell walls dictates that cell wall metabolism is an important feature of the PhoPR-mediated phosphate limitation response. Here we report the expression profiles of cell-envelope-associated and PhoPR regulon genes, determined by live cell array and transcriptome analysis, in exponentially growing and phosphate-limited *B. subtilis* cells. Control by the WalRK two-component system confers a unique expression profile and high level of promoter activity on the genes of its regulon with *yocH* and *cwlO* expression differing both qualitatively and quantitatively from all other autolysin-encoding genes examined. The activity of the PhoPR two-component system is restricted to the phosphate-limited state, being rapidly induced in response to the cognate stimulus, and can be sustained for an extended phosphate limitation period. Constituent promoters of the PhoPR regulon show heterogeneous induction profiles and very high promoter activities. Phosphate-limited cells also show elevated expression of the actin-like protein MreBH and reduced expression of the WapA cell wall protein and WprA cell wall protease indicating that cell wall metabolism in this state is distinct from that of exponentially growing and stationary-phase cells. The PhoPR response is very rapidly deactivated upon removal of the phosphate limitation stimulus with concomitant increased expression of cell wall metabolic genes. Moreover expression of genes encoding enzymes involved in sulphur metabolism is significantly altered in the phosphate-limited state with distinct perturbations being observed in wild-type 168 and AH024 (Δ *phoPR*) cells.

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INTRODUCTION

The cell envelope of a Gram-positive bacterium such as *Bacillus subtilis* comprises the cell wall and cytoplasmic membrane and the space located between them. The cell wall is a 3D mesh composed of peptidoglycan to which an anionic polymer such as teichoic acid is covalently attached. Teichoic acid covalently linked to lipid (lipoteichoic acid) is also present at the outer face of the plasma membrane and there are a host of proteins present in all three compartments (Vollmer *et al.*, 2008; Weidenmaier & Peschel, 2008). The cell wall determines the shape of the

bacterium, forms a protective barrier between the cell and the external environment and creates a buffer zone with a distinctive microenvironment in which cell envelope synthetic and maintenance activities can be executed.

The first stage of peptidoglycan synthesis occurs cytoplasmically where Lipid II [undecaprenyl-pyrophosphoryl-MurNAc-(pentapeptide)-GlcNAc] is synthesized by the MurAA, MurBCDEFG and MraY enzymes. Lipid II is then flipped across the membrane and incorporated into nascent cell wall peptidoglycan by the transglycosylase and transpeptidase activities of the penicillin-binding proteins (see Bhavsar & Brown, 2006 for review). Cell wall hydrolases (autolysins) cleave mature peptidoglycan to allow insertion of new material into the cell wall during cell elongation and division. The CwlO and LytE endopeptidase-type autolysins play a crucial role in cell wall synthesis since cells devoid of both autolysins fail to elongate and divide (Bisicchia *et al.*, 2007). Expression of these two autolysins, together with YocH (an amidase-type autolysin), YdjM (a cell-wall-associated protein) and FtsZ, are controlled by the WalRK (YycFG) two-component system (TCS), which is activated by a signal emanating from the divisome (Fukuchi *et al.*, 2000; Howell *et al.*, 2003;

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Abbreviations: LCA, live cell array; P_{act} , promoter activities; TCS, two-component system.

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Bisicchia *et al.*, 2007; Dubrac *et al.*, 2008; Fukushima *et al.*, 2008, 2011). Teichoic acid is synthesized by the activities of the TagABDEFGHO and Mna proteins (see Bhavsar & Brown, 2006 for review). Like peptidoglycan synthesis, teichoic acid polymers are assembled cytoplasmically by the TagABDEFO enzymes using the undecaprenol phosphate lipid carrier, are exported by the TagGH ABC-type transporter and attached to peptidoglycan by enzymes yet to be identified. Expression of the TagABDEFGHO enzymes is constitutive in exponentially growing cells. The synthetic enzymes for both polymers appear to be assembled in individual complexes whose formation and cellular location is influenced by membrane-spanning proteins (e.g. MreCD) and by cytoplasmically located helical filaments composed of actin-like (MreB, Mbl and MreBH) proteins (Carballido-López & Formstone, 2007; Formstone *et al.*, 2008; Kawai *et al.*, 2009a). Autolysin localization and function are also influenced by these actin-like proteins and by teichoic acid (Carballido-López *et al.*, 2006; Yamamoto *et al.*, 2008).

When *B. subtilis* cells are exposed to phosphate-limiting conditions, teichoic acid synthesis is repressed and teichuronic acid (a non-phosphate-containing anionic polymer) synthesis is activated, a response that is executed by the PhoPR TCS (Hulett, 2002). Importantly peptidoglycan synthesis continues at a significant rate during this change in anionic polymer composition (Bisicchia *et al.*, 2007). Teichoic acid and peptidoglycan metabolism in *B. subtilis* are linked in two crucial ways: (i) teichoic acid is covalently bound to peptidoglycan and (ii) the subunits of peptidoglycan (Lipid II) and teichoic acid (undecaprenyl-pyrophosphoryl-GlcNAc-ManNAc-polyglycerol phosphate) synthesis are both assembled intracellularly using the same pool of undecaprenol phosphate (Bhavsar & Brown, 2006; Bouhss *et al.*, 2008; Weidenmaier & Peschel, 2008; Swoboda *et al.*, 2010). Thus coordinate regulation of peptidoglycan

and anionic polymer synthesis must occur during exponential growth and under phosphate-limiting conditions.

An important feature of the coordinated regulation of peptidoglycan and teichoic acid synthesis emanates from structural similarities and functional overlap of the WalRK and PhoPR TCS (Howell *et al.*, 2006; Bisicchia *et al.*, 2007, 2010). Our model (Fig. 1) proposes that the WalRK TCS senses cell growth through septal location of WalK and is activated by a signal emanating from the divisome (Fukushima *et al.*, 2008, 2011). WalR-P activates expression of autolysins (CwlO, YocH and LytE), cell-wall-associated (YdjM) and cell division (FtsZ) proteins while repressing autolysin-modulating (YjeA) and -inhibiting (IseA) activities thereby promoting cell wall synthesis during cell elongation (Bisicchia *et al.*, 2007; Fukushima *et al.*, 2008, 2011). During phosphate limitation, the WalK signal is lowered or absent due to reduced growth rate, but peptidoglycan synthesis is still required (Bisicchia *et al.*, 2007). Therefore in phosphate-limited cells, PhoPR not only induces expression of phosphate-scavenging enzymes and effects a change in anionic polymer synthesis but also plays a role in peptidoglycan synthesis by controlling expression of WalRK regulon autolysins (Bisicchia *et al.*, 2010). The latter function is achieved through phosphorylation of WalR by the activated non-cognate PhoR kinase and through PhoP-P-mediated activation of WalRK regulon genes (Howell *et al.*, 2006; Bisicchia *et al.*, 2010). Therefore the activities of WalRK and PhoPR together ensure that peptidoglycan synthesis is maintained and coordinately regulated with anionic polymer metabolism under phosphate-limiting conditions.

In this study we combine transcriptome and live cell array (LCA) analysis to establish the changes that occur in gene expression when cells enter the phosphate-limited state. We focus in particular on cell envelope metabolism, on the roles

Fig. 1. Model of the relationship between the WalRK (YycFG) and PhoPR regulons during growth and phosphate limitation in *B. subtilis*. Black signifies the activities of the WalRK TCS and grey signifies the activities of the PhoPR TCS. The daggers symbolize the signal that activates each histidine kinase. Thin lines represent phosphorylation reactions while thick lines indicate control of (+, activation; -, inhibition) gene expression. Broken lines signify activities of PhoPR carried out on the WalRK TCS or regulon.

of the WalRK and PhoPR TCS and on the kinetics of the PhoPR-mediated response to phosphate limitation and replenishment, respectively. We show that entry into the phosphate-limited state triggers extensive changes in gene expression including significant perturbation of sulphur metabolism. Distinctive expression profiles of genes encoding cytoskeletal proteins and wall-associated proteins are observed, suggesting that cell envelope structure and metabolism are specifically adapted to the phosphate-limited state. Moreover the very rapid resumption of growth and the high level of cell envelope gene expression that occur when phosphate is added to phosphate-starved cultures of AH024 (Δ phoPR) cells show that the PhoPR TCS adapts cells to phosphate limitation but is not required to maintain viability in the phosphate-limited state.

METHODS

Bacterial strains and growth conditions. All strains used in this study (see Supplementary Table S1, available with the online version of this paper) were maintained and propagated in Luria–Bertani (LB) medium. *Escherichia coli* TG-1 was transformed under standard procedures and was used for all plasmid constructions (Sambrook *et al.*, 1989). *B. subtilis* 168 and a phoPR null mutant (AH024, Howell *et al.*, 2003) were used for transcriptome analysis. The *B. subtilis* tryptophan prototrophic BSB168 (Jules *et al.*, 2009) and AH024 (Δ phoPR) were transformed using standard procedures (Anagnostopoulos & Spizizen, 1961). For transcriptome analysis and LCA experiments, *B. subtilis* strains were grown in a low-phosphate defined medium (LPDM) or in a high-phosphate defined medium (HPDM) (Müller *et al.*, 1997). Media were supplemented with antibiotics at the following concentrations as required: ampicillin, 100 µg ml⁻¹; spectinomycin, 100 µg ml⁻¹; erythromycin, 3 µg ml⁻¹.

Primer design, and plasmid and strain construction. The list of operons investigated and the constituent genes of each operon are given in Supplementary Table S2 (available with the online version of this paper). Of 135 promoters examined, 109 promoters yielded promoter activities above a 25 unit background threshold. Promoter regions were operationally defined as the entire intergenic region of interest excluding the 18 nt preceding the start codon of the first cistron (to exclude the endogenous ribosome-binding site). To improve plasmid integration by single crossover, the minimum size of each promoter-containing fragment was 400 bp. Thus for intergenic regions smaller than 400 bp, the 3'-end of the upstream gene was included in the amplified fragment. All integrations were non-mutagenic (Botella *et al.*, 2010). Primers designed to specifically amplify each promoter region had ligation-independent cloning (LIC) tails (5'-CCGCGGGC-TTCCAGC-3' forward primer and 5'-GTTCTCTTCCACC-3' reverse primer) added for high throughput cloning. Promoter fragments were generated from genomic *B. subtilis* 168 DNA by PCR using the primers listed in Supplementary Table S3 and were cloned into pBaSysBioII as described by Botella *et al.* (2010). The resulting plasmids (see Supplementary Table S4) were sequenced using primer GFP1 and then transformed into BSB168 and AH024 (Δ phoPR) recipient strains. Three *B. subtilis* clones from each transformation were isolated and used as biological replicates in LCA experiments. Strains are listed in Supplementary Table S1.

***B. subtilis* LCA methodology.** *B. subtilis* strains were grown overnight in a 96-well plate (BD Falcon 353075) at 37 °C in 100 µl HPDM supplemented with appropriate antibiotics. These cultures were used to inoculate 100 µl cultures of HPDM or LPDM (with no

antibiotic) to OD₆₀₀ 0.05 in a 96-well plate (Greiner 655180). The resulting culture plates were incubated with lids in a Synergy multimode microplate reader (BioTek) for 10 h with constant shaking (slow) at 37 °C. To determine background fluorescence, six wells were inoculated with the corresponding recipient strain (BSB168 or AH024) on every plate. Fluorescence (excitation 485/20 nm, emission 528/20 nm) and OD₆₀₀ were measured every 10 min throughout the experiment. For phosphate injection experiments, 5 µl of a 73 mM phosphate buffer solution was added to LPDM cultures thereby converting the phosphate concentration to that of HPDM.

Promoter activities were calculated as described previously (Botella *et al.*, 2010) with the following modifications: background fluorescence of one of the six replicates of parental strain 168 or AH024 (Δ phoPR) cultures was subtracted from the raw fluorescence of all *gfp* fusion strains at the same OD₆₀₀ and from the background fluorescence of the other five replicates of parental strains *B. subtilis* strain 168 or AH024 (Δ phoPR). The value of the remaining background fluorescence of the non-fusion-containing parental strains [*B. subtilis* strain 168 or AH024 (Δ phoPR)] at each time point was then subtracted from the fusion-containing strains at the same time point. Growth and background-corrected fluorescence profiles of all cultures were aligned according to the *T*₀, calculated as the time at which the culture stops growing exponentially using the Heat Map Generator web software. Promoter activities (*P*_{act}) were calculated by taking the derivative of the fluorescence divided by the OD₆₀₀ [(ΔGFP/ΔTime)/OD₆₀₀] at each time point and smoothed by averaging the activity values of three consecutive time points as described in detail by Botella *et al.* (2010). At least three independent isolates of each fusion-containing strain (three biological replicates) were analysed and at least two *P*_{act} determinations of each isolate were performed on two different Biotek microplate readers by different operatives under all conditions (at least two technical replicates). Importantly, by quantifying the *P*_{act} of cell wall protein and PhoPR regulon promoters in this standard manner, the promoter strengths and kinetics of activity for the 135 cell-wall-associated genes can be compared directly. Promoter activities were uploaded to the Heat Map Generator software platform for generation of heat maps and for analysis. To avoid artefacts, data collected during the first 20 min of each growth curve were not used for promoter activity determination and *P*_{act} values below 25 units were considered to be background and assigned a value of zero.

Transcriptome analysis. Strains 168 (wild-type) and AH024 (Δ phoPR) were grown in LPDM (Müller *et al.*, 1997) and cells were harvested by centrifugation (1 min at 8000 r.p.m.) during exponential growth (OD₆₀₀ 0.25) and at 2 h after the outset of the phosphate-limitation-induced stationary phase. Both strains were also grown in HPDM (Müller *et al.*, 1997) and harvested during exponential growth (OD₆₀₀ 0.25). Three biological repeats were performed for each strain and condition. RNA isolation was carried out as described previously (Eymann *et al.*, 2002). The microarray experiment was performed utilizing the tiling array BaSysBio Bsub T1 and cDNA synthesis and hybridization were performed as described in Rasmussen *et al.* (2009). The sequence similarity search tool Blat (Kent, 2002) was applied to remap the probe sequences of the tiling chip to the latest version of the *B. subtilis* genome. Sequences without a unique and perfect match were discarded. This reduced the number of probes from 383 149 to 369 186. Expression data from the reduced set of probes were normalized using the normalize.quantiles method (Gentleman *et al.*, 2004) as implemented in Bioconductor version 2.6 (Bolstad *et al.*, 2003). The values from probes that lie entirely within the border of a gene were then merged. Statistical significance of the fold changes was calculated through the linear analysis of microarray data (limma) empirical Bayes method (Smyth, 2004) with Benjamini–Hochberg (Hochberg & Benjamini, 1990) correction for multiple testing. All raw data (pair

files) as well as processed data have been submitted to the GEO database under accession no. GSE23164.

Heat Map Generator. To generate heat map images a modified version of a tool called ArrayPipe (Hokamp *et al.*, 2004) was used. ArrayPipe serves mainly for the analysis of microarray data but also includes a web-based spreadsheet for online data mining. After modifications to the output format of the HTML table and colouring the cells according to the values contained therein, the data presentation turns from text format into an online heat map that can also be saved in PDF and PNG format. A freely available web-based tool dedicated to the generation of heat maps from LCA data has been set up at <http://bioinf.gen.tcd.ie/HMG/>. The Heat Map Generator provides methods for processing tab-delimited data files, including smoothing, scaling and merging of replicates. The results are then transferred into the ArrayPipe online spreadsheet with preset parameters so that a heat map is created. The output in the form of a web page can be password-protected, bookmarked and shared with others. It can also be used for further data manipulation, such as clustering and attachment of annotations. Detailed documentation for users is available online.

RESULTS AND DISCUSSION

Validation of promoter activity profiles determined by LCA technology

The complementary approaches of LCA and transcriptome analyses using a high-density (tiled) microarray were adopted to establish the changes in gene expression (P_{act} , promoter activity and RNA abundance) that occur under our experimental conditions (Botella *et al.*, 2010; Rasmussen *et al.*, 2009). The high throughput nature of LCA analysis has allowed us to determine, with a time resolution of 10 min, the P_{act} of 135 cell wall protein promoters and PhoPR regulon promoters that direct expression of a total of 300 genes during growth in HPDM, in LPDM and during phosphate replenishment of phosphate-limited cells (Fig. 2 and Supplementary Figs S1 and S2, available with the online version of this paper). Results were visualized in a heat map format generated by Heat Map Generator, a web-based tool developed by us for this study (available at <http://bioinf.gen.tcd.ie/HMG/>). The numerical value of the maximum P_{act} of each transcriptional fusion in each growth condition is shown to the right of each row (max value, Fig. 2 and Supplementary Figs S1 and S2). By referring to the colour legend (showing the fraction of the maximum P_{act} represented by each colour), numerical P_{act} values for each fusion can be established at all growth stages and the strengths of different promoters can be compared. The combined LCA/transcriptome approach allows promoter activities to be correlated with relative RNA transcript abundance – this correlation is valid for the two time points at which RNA was sampled (exponentially growing and phosphate-limited cells, Fig. 2b, c and Supplementary Fig. S2). Differences in relative RNA transcript abundance between exponentially growing and phosphate-limited cells are shown for wild-type (Fig. 2b and Supplementary Fig. S2) and AH024 ($\Delta phoPR$) (Fig. 2c) cultures. To validate the P_{act} profiles established by LCA in this study, we compared the reported expression of several genes that have distinctive

profiles (*murAA*, *murAB*, *cwlS* and genes of the WalRK and PhoPR regulons) with the kinetics of P_{act} established in this study. Previous studies show (i) that *murAA* expression (RNA accumulation) is confined to exponential growth and is turned off when cells enter stationary phase while *murAB* is expressed (RNA accumulation) during stationary phase (Kock *et al.*, 2004); (ii) that *cwlS* is expressed (RNA and LacZ accumulation) at significant levels during late exponential growth and early stationary phase (Fukushima *et al.*, 2006); (iii) that expression (RNA accumulation) of WalRK regulon genes *yocH*, *cwlO* and *ydjM* is highest immediately after inoculation into HPDM medium but decreases to a low level thereafter even while cells are growing exponentially (Howell *et al.*, 2003, 2006) and (iv) that PhoPR regulon genes are induced (RNA and LacZ accumulation) upon phosphate limitation (Allenby *et al.*, 2005; Hulett, 2002 and references therein; Prágai & Harwood, 2002). The P_{act} profiles for these genes determined using transcriptional *gfp* fusions in this study (Fig. 2, Supplementary Figs S1 and S2) are consistent with their RNA and/or LacZ accumulation profiles established in the cited studies, supporting the view that the P_{act} profiles accurately reflect promoter activity.

Population heterogeneity of promoter fusion expression

The promoter activity values determined in this study reflect total GFP production in the cell population. However, the activity of a promoter expressed heterogeneously in the cell population would be underestimated in this analysis. We therefore examined GFP production microscopically in 58 strains during exponential growth and phosphate limitation. The chosen strains represent all the categories listed in Fig. 2(a and b), eight of the strains in which GFP expression was below the threshold of detection and all genes whose expression is controlled by a regulator in addition to, or other than, SigA. Uniform GFP expression of PhoPR-regulated genes was observed in phosphate-limited cells. The five promoter fusions (*dltA*, *lytA*, *lytD*, *lytF* and *yjbJ*), whose expression is controlled by SigD, are expressed at relatively low levels (Fig. 2b, c). We observed only low fluorescence in these populations microscopically and did not detect population heterogeneity, probably due to the low expression level. Since heterogeneity of SigD-regulated promoters has been documented (e.g. the *hag* gene) it is likely that expression of these five genes is heterogeneous within these populations, implying that their maximum promoter activities reported here are an underestimate of that present in expressing cells. Population heterogeneity was not observed in any of the other strains examined.

General features of cell envelope metabolism during growth and in the phosphate-limited state

The related WalRK and PhoPR TCs play key roles in effecting the differential patterns of cell envelope gene expression that occur when cells enter the phosphate-limited state (Hulett, 2002; Prágai & Harwood, 2002; Howell *et al.*,

Fig. 2. Heat map representation of the activities of promoters that direct expression of operons encoding cell-envelope-related genes. Growth and promoter activity (P_{act}) of cell-envelope-related genes were determined in *B. subtilis* wild-type cells using LCA technology. A representative growth curve is shown at the top of each figure showing the boundaries between lag phase and exponential growth (vertical broken line) and between exponential growth and stationary phase or phosphate limitation (vertical continuous line), respectively. Each promoter is named after the first gene in the operon and is shown on the left hand side of the map while the maximum activity (max) attained by each promoter in each growth curve is given to the right. The promoter activity at each 10 min interval was normalized to the maximum value (max) observed in each growth curve and then assigned a colour as detailed in the colour legend below the map. The open triangles in (b) and (c) signify the points at which RNA was harvested for transcriptome analysis. The fold increase (positive values) or decrease (negative values) in the RNA transcript level of each operon (values are an average of the values for each cistron in the operon) in phosphate-limited cells relative to exponentially growing cells is shown (fold change). (a) Activities of 109 promoters of *B. subtilis* wild-type cells growing in HPDM. (b) Activities of 109 promoters of *B. subtilis* wild-type cells (left hand side) growing in LPDM and (right hand side) upon addition of phosphate to cells that were phosphate starved for 3 h. (c) Activities of 106 promoter of *B. subtilis* AH024 ($\Delta phoPR$) cells (left hand side) growing in LPDM and (right hand side) upon addition of phosphate to cells that were phosphate starved for 3 h. Categories: A, transcriptional regulators; B, cell division; C, cell shape; D, cell and envelope stress; E, Lipid II synthesis; F, penicillin-binding proteins; G, WalRK regulon; H, autolysins; I, teichoic, minor teichoic and lipoteichoic acid; J, wall proteins; K, wall recycling; L, phospholipid synthesis; M, PhoPR regulon. (a) is on this page; (b) and (c) are on the following pages.

2003, 2006; Allenby *et al.*, 2005; Bisicchia *et al.*, 2007, 2010). We therefore established promoter activity and relative mRNA abundance for a comprehensive list of cell-envelope-associated and PhoPR regulon genes (of 135 promoters tested, 109 had P_{act} above the applied threshold of 25 arbitrary GFP units). Our results are presented in two formats to facilitate analysis of this large dataset. The first format groups the genes under 13 headings (Fig. 2) each of which is a subprocess of cell wall metabolism in *B. subtilis*: transcriptional regulators (A); cell division (B); cell shape (C); cell and envelope stress (D); Lipid II synthesis (E); penicillin-binding proteins (F); WalRK regulon (G); autolysins (not controlled by WalRK) (H); teichoic, minor teichoic and lipoteichoic acid (I); wall proteins (J); wall recycling (K); phospholipid synthesis (L); and the PhoPR regulon (M). The second format clusters the genes according to their expression profiles (Supplementary Figs S1 and S2). In general, P_{act} of the genes encoding enzymes for Lipid II, lipoteichoic acid and phospholipid synthesis, together with those encoding cell division and penicillin-binding proteins was commensurate with the growth status of the culture, i.e. P_{act} values were maximal in exponentially growing cells, lower (ranging from ~20–80 % of maximum activity) in phosphate-limited wild-type cells and greatly reduced (<10 %, sometimes reaching ~20 % sporadically) in phosphate-limited AH024 ($\Delta phoPR$) cells. These observations are in accordance with the observed growth and peptidoglycan synthesis rates reported (Bisicchia *et al.*, 2007). Although phosphate limitation generates a general stress [expression of the SigB-regulated gene *ctc* is induced in wild-type (180 units) and to an even greater extent in AH024 ($\Delta phoPR$) cells (309 units)] it did not appear to engender a cell envelope stress, since genes diagnostic of ECF-type sigma factor activation, LiaRS, or those encoding HtrA-type proteases were not significantly induced in wild-type or more notably even in AH024 ($\Delta phoPR$) cells.

WalRK- and PhoPR-activated promoters have high P_{act} values

Maximum P_{act} for the great majority of the 109 promoters ranged from 0 to 700 units for exponentially growing cells and from 0 to 500 units for phosphate-limited cells (Fig. 3). It is striking that the maximum P_{act} of most WalRK- and PhoPR-activated promoters [the exceptions being *cwlO* (359 units) and *yurI* (406 units) in phosphate-limited cells] was significantly higher than the upper limit of these ranges (Fig. 3). Thus WalRK- and PhoPR-mediated control directs a very high level of promoter activity in addition to making expression of their regulons sensitive to the activating stimuli of their respective sensor kinases.

Expression of WalRK regulon autolysins YocH and CwlO is unique among the cohort of autolysins investigated

Promoter activity of eight known autolysin-encoding genes was examined in this study: *cwlS* (encoding an

Fig. 3. Distribution of promoter strengths of the 109 promoters that direct expression of operons encoding cell-envelope-related genes in *B. subtilis*. Genes are categorized into bins according to the maximum promoter activity (P_{act}) attained during growth in HPDM (a) and LPDM (b). The number of promoters in each bin is shown on the y-axis while the promoter activity range (<100 signifies P_{act} in the range 0–100; <200 signifies P_{act} in the range 101–200 etc.) is shown on the x-axis. Promoters whose expression is controlled by the WalRK TCS are shown in bold type, while promoters controlled by the PhoPR TCS are underlined.

endopeptidase), the major cell autolysin *lytC* (*lytA* promoter, encoding an amidase), *lytD* (encoding an *N*-acetylglucosaminidase), *lytF* (encoding a γ -D glutamate meso DAP mureopeptidase) and *lytG* (encoding a glucosaminidase), and *cwlO*, *yocH* and *lytE*, which are members of the WalRK regulon. The profile and expression level of two WalRK regulon autolysins (CwlO and YocH) differed greatly from those of the other autolysins examined (regulation of *LytE* expression is complex and not solely dependent on WalRK): maximum P_{act} levels of CwlO and YocH were observed immediately upon inoculation in the lag phase of the growth cycle while P_{act} for the other autolysins remained at <10 % of maximum during this period. The maximum promoter activities of *cwlO* (359 units) and *yocH* (2623 units) were significantly higher than those of the other autolysins (*cwlS*, 56 units; *lytA*, 134 units; *lytD*, 38 units; *lytF*, 20 units and *lytG*, 60 units), whose maximum P_{act} values were observed during

Table 1. Differences in the relative RNA abundance between phosphate-limited wild-type and AH024 (Δ *phoPR*) cells

Only differences of fivefold or greater are shown. For all genes with difference values greater than twofold see Supplementary Tables S7–S10.

Gene	Difference	Fold change		Function*	Product*
		WT	AH024		
Group A. Genes expressed at significantly higher levels in the wild-type					
<i>glpQ</i>	198.2	76.6	−2.6	Glycerol-3-phosphate utilization	Glycerolphosphate diester phosphodiesterase
<i>tuaB</i>	191.1	205.9	1.1	Biosynthesis of teichuronic acid	Unknown
<i>tuaC</i>	176.6	171.5	−1.0	Biosynthesis of teichuronic acid	Sugar transferase
<i>pstA</i>	169.6	180.0	1.1	High-affinity phosphate uptake	Phosphate ABC transporter (permease)
<i>pstC</i>	167.8	178.2	1.1	High-affinity phosphate uptake	Phosphate ABC transporter (permease)
<i>ydhF</i>	165.4	184.7	1.1	Unknown	Unknown
<i>tuaD</i>	165.1	162.5	−1.0	Biosynthesis of teichuronic acid	UDP glucose 6-dehydrogenase
<i>tuaE</i>	160.2	132.5	−1.2	Biosynthesis of teichuronic acid	Unknown
<i>phoA</i>	152.6	107.0	−1.4	Acquisition of phosphate upon phosphate starvation	Alkaline phosphatase A
<i>pstBA</i>	144.4	158.4	1.1	High-affinity phosphate uptake	Phosphate ABC transporter (ATP-binding protein)
<i>pstBB</i>	140.3	154.8	1.1	High-affinity phosphate uptake	Phosphate ABC transporter (ATP-binding protein)
<i>phoB</i>	136.9	162.3	1.2	Acquisition of phosphate upon phosphate starvation	Alkaline phosphatase III
<i>tuaA</i>	131.8	199.2	1.5	Biosynthesis of teichuronic acid	Lipid carrier sugar transferase
<i>tuaG</i>	123.2	116.0	−1.1	Biosynthesis of teichuronic acid	Sugar transferase
<i>tuaF</i>	118.9	109.2	−1.1	Biosynthesis of teichuronic acid	Unknown
<i>phoD</i>	107.5	98.6	−1.1	Acquisition of phosphate upon phosphate starvation	Phosphodiesterase/alkaline phosphatase
<i>tuaH</i>	103.5	112.3	1.1	Biosynthesis of teichuronic acid	Sugar transferase
<i>pstS</i>	98.3	109.7	1.1	High-affinity phosphate uptake	Phosphate ABC transporter (binding protein)
<i>tatAD</i>	96.9	83.1	−1.2	Protein secretion	Component of the TAT pathway
<i>ykoL</i>	61.2	91.8	1.5	Unknown	Unknown
<i>yurI</i>	51.9	61.6	1.2	Extracellular RNA degradation	Extracellular RNase Bsn
<i>nasD</i>	27.9	13.1	−2.1	Utilization of nitrite as nitrogen source	Assimilatory nitrite reductase (subunit)
<i>tatCD</i>	27.4	27.2	−1.0	TAT protein secretion	Component of the TAT pathway
<i>yycO</i>	23.2	25.8	1.1	Unknown	Unknown
<i>yfkN</i>	23.2	12.5	−1.9	Probably phosphate acquisition	2',3' cyclic nucleotide phosphodiesterase, 2' (or 3') nucleotidase and 5' nucleotidase
<i>yycP</i>	13.6	13.1	−1.0	Unknown	Unknown
<i>sspC</i>	9.3	11.1	1.2	Protection of spore DNA	Small acid-soluble spore protein (minor alpha/beta-type SASP)
<i>treP</i>	8.9	10.7	1.2	Trehalose uptake and phosphorylation	Trehalose-specific phosphotransferase system, EIIBC component
<i>copA</i>	8.1	6.4	−1.3	Copper export	Copper transporting ATPase
<i>nasE</i>	7.8	5.5	−1.4	Utilization of nitrite as nitrogen source	Assimilatory nitrite reductase (subunit)
<i>yxiO</i>	7.5	15.0	2.0	Unknown	Unknown
<i>yjdB</i>	6.5	14.4	2.2	Unknown	Unknown
<i>guaC</i>	6.4	4.8	−1.3	Purine salvage and interconversion	GMP reductase
<i>ctaO</i>	5.7	3.3	−1.7	Haem biosynthesis	Haem O synthase (minor enzyme)
<i>treA</i>	5.6	6.1	1.1	Trehalose utilization	Phospho-alpha-glucosidase
<i>fnr</i>	5.4	5.2	−1.0	Regulation of anaerobiosis, fermentation and overflow metabolism	Transcription regulator (Crp family)
Group B. Genes expressed at significantly lower levels in the wild-type					
<i>tagA</i>	−11.5	−5.4	2.1	Biosynthesis of teichoic acid	UDP-N-acetyl-D-mannosamine transferase
<i>tagB</i>	−8.7	−7.5	1.2	Biosynthesis of teichoic acid	Putative CDP-glycerol: glycerol phosphate glycerophosphotransferase
<i>tcyP</i>	−8.1	−9.2	−1.1	Cysteine uptake	Cysteine transporter, membrane protein
<i>sumT</i>	−5.5	−2.8	2.0	Sirohaem biosynthesis, sulphite reduction	Probable uroporphyrin-III C-methyltransferase
<i>cysC</i>	−5.3	−3.0	1.8	Sulphate reduction and activation	Adenylyl-sulphate kinase

Table 1. cont.

Gene	Difference	Fold change		Function*	Product*
		WT	AH024		
Group C. Genes expressed at significantly higher levels in AH024					
<i>yyrT</i>	−26.0	−1.7	14.9	Methionine salvage	Putative AdoMet-dependent methyltransferase
<i>mccB</i>	−22.0	−1.6	13.7	Methionine-to-cysteine conversion	Cystathionine lyase/homocysteine gamma-lyase
<i>mccA</i>	−19.0	−1.5	12.8	Methionine-to-cysteine conversion	O-acetylserine-thiol-lyase
<i>gapB</i>	−14.8	−1.4	10.6	Anabolic enzyme in gluconeogenesis	Glyceraldehyde-3-phosphate dehydrogenase 2
<i>yzkW</i>	−11.4	1.3	15.2	Control of arginine metabolism and glycolysis	Small regulatory RNA/regulatory peptide
<i>yqkK</i>	−9.5	4.8	46.2	Unknown	Unknown
<i>ywsA</i>	−7.8	1.2	9.3	Unknown	Unknown
<i>glcD</i>	−6.4	1.2	7.9	Unknown	Unknown
<i>yopM</i>	−6.3	−1.1	5.7	Unknown	Unknown
Group D. Genes expressed at significantly lower levels in AH024					
<i>cysJ</i>	20.2	1.1	−19.2	Sulphite reduction	Sulphite reductase (NADPH ₂)
<i>cysI</i>	15.7	1.2	−13.3	Sulphite reduction	Sulphite reductase (NADPH ₂)
<i>cheB</i>	7.6	−1.6	−12.0	Motility and chemotaxis	MCP-glutamate methyltransferase/two-component response regulator-like
<i>pyrB</i>	7.1	−5.3	−37.9	Pyrimidine biosynthesis	Aspartate carbamoyltransferase
<i>cheA</i>	7.1	−1.5	−10.6	Chemotactic signal modulator	Two-component sensor kinase
<i>pyrC</i>	7.0	−7.2	−50.3	Pyrimidine biosynthesis	Dihydroorotase
<i>pyrAA</i>	6.9	−8.9	−61.7	Pyrimidine biosynthesis	Carbamoyl-phosphate synthetase (glutaminase subunit)
<i>pyrP</i>	6.4	−5.8	−37.0	Uracil transport in/out via proton symport	Uracil permease
<i>yqgB</i>	6.1	1.5	−4.2	Unknown	Unknown
<i>ylxH</i>	5.9	−1.6	−9.7	Self-resistance to surfactin and swarming motility	Swarming motility protein
<i>flhA</i>	5.7	−1.5	−8.4	Movement and chemotaxis	Flagella-associated protein
<i>cheC</i>	5.7	−1.4	−8.2	Motility, chemotaxis	CheY-P phosphatase
<i>pyrAB</i>	5.6	−10.2	−57.2	Pyrimidine biosynthesis	Carbamoyl-phosphate synthetase (catalytic subunit)
<i>pyrE</i>	5.5	−10.6	−58.2	Pyrimidine biosynthesis	Orotate phosphoribosyltransferase
<i>purA</i>	5.5	−1.6	−8.9	Purine biosynthesis	Adenylosuccinate synthetase
<i>flhF</i>	5.2	−1.6	−8.2	Placement and assembly of polar flagella	Signal recognition particle-GTPase
<i>rplJ</i>	5.2	−3.7	−19.2	Translation	Ribosomal protein L10 (BL5)
<i>pyrK</i>	5.1	−9.9	−50.8	Pyrimidine biosynthesis	Dihydroorotic acid dehydrogenase (electron transfer subunit)

*Functions and products are taken from SubtiWiki (<http://www.subtiwiki.uni-goettingen.de/wiki/>).

exponential growth. These expression features suggest that CwlO and YocH have significantly different functions to the other cellular autolysins examined. Moreover the differing profiles and levels of the remaining known and putative autolysins show that a complex array of autolysin activities and levels is required for normal cell wall metabolism.

MreBH expression is distinct from its paralogues MreB and Mbl

The actin-like proteins MreB, Mbl and MreBH have overlapping cellular functions and play an important role

(together with MreCD and MinCD, which are encoded in the *mreB* operon) in positioning the cell wall synthetic machinery and in cell shape (Jones *et al.*, 2001; Leaver & Errington, 2005; Carballido-López *et al.*, 2006; Kawai *et al.*, 2009a, b). Our results show that *mreBH* expression differs from that of *mreB* and *mbl* in phosphate-limited cells, suggesting that it performs distinct functions under these conditions (Fig. 2 and Supplementary Figs S1 and S2). The maximum P_{act} of the *mreB* and *mbl* operons was ~330 units in wild-type cells growing exponentially in HPDM (Fig. 2a) and LPDM (Fig. 2b). The P_{act} of both operons continued at ~50–100 % (*mreB*) and 20–40 % (*mbl*) of maximum activity in stationary-phase cells (Fig.

2a) but was reduced to <10 % of maximal activity in phosphate-limited cells (Fig. 2b). Moreover the abundance of *mreB* and *mbl* RNA transcripts was approximately three- to fourfold lower in phosphate-limited cells than in exponentially growing cells, consistent with these P_{act} profiles (Fig. 2c). Expression of *mreBH* differed from that of *mreB* and *mbl* under these conditions: in HPDM, expression was initially high (P_{act} 343 units) in lag phase cells, but fell to a very low level (<5 %) in exponentially growing and stationary-phase cells (Fig. 2a). In cells growing exponentially in LPDM, *mreBH* expression is significantly lower (P_{act} ~53 units) than that of *mreB* and *mbl* but is maintained at 50–100 % of maximum activity throughout the phosphate limitation period (Fig. 2b). These promoter activity profiles are supported by the observation that *mreBH* RNA transcripts increased approximately twofold whereas *mreB* and *mbl* transcripts decreased three- to fourfold upon phosphate limitation (Fig. 2b). Expression of *mreBH* was also distinguished from that of *mreB* and *mbl* upon addition of phosphate to phosphate-limited cells. The P_{act} of *mreB* and *mbl* were immediately induced to maximum levels and remained at 50–100 % of maximum activity for up to 150 min whereas the P_{act} of *mreBH* was reduced to <5 % of maximum activity within 60 min and remained at this level for the remainder of the experiment. These expression profiles distinguish the paralogous actin-like genes from each other and suggest that MreB and Mbl predominate in exponentially growing cells whereas MreBH may predominate in phosphate-limited cells. Soufo & Graumann (2010) show that MreB, Mbl and MreBH have different architectures but influence each other's architecture when co-expressed. Thus the altered proportions of actin-like proteins indicated by these expression studies suggest that cell morphogenesis, cell wall metabolism or placement of the peptidoglycan synthetic machinery may differ considerably in exponentially growing and phosphate-limited *B. subtilis* cells (Daniel & Errington, 2003; Divakaruni *et al.*, 2005, 2007; Carballido-López & Formstone, 2007).

Expression of WapA and WprA is greatly reduced in phosphate-limited cells

Expression of the wall-associated protein WapA (Serizawa *et al.*, 2005) and wall protease WprA (Babé & Schmidt, 1998) differs significantly between exponentially growing and stationary-phase cells (Fig. 2a) and phosphate-limited cells (Fig. 2b). The P_{act} of *wapA* was maximal (~711 units) throughout exponential growth in HPDM and continued at 20–50 % of this activity for a considerable period of the stationary phase (Fig. 2a). However, while the P_{act} of *wapA* in wild-type cells was also maximal during exponential growth in LPDM (~470 units), it was reduced to <5 % of maximum activity at the onset of, and throughout, phosphate limitation (Fig. 2b). The expression profiles of *wprA* are similar to those of *wapA* although P_{act} levels are considerably lower (74–175 units, Fig. 2b). Moreover P_{act}

values for both *wapA* and *wprA* were restored to maximum upon phosphate replenishment of phosphate-limited cells (Fig. 2b, c). Transcriptome analysis supports these P_{act} profiles: the relative abundances of *wapA* and *wprA* transcripts in phosphate-limited wild-type cells was 20- and 16-fold lower than in exponentially growing cells (Fig. 2b, c, and see Supplementary Table S5, available with the online version of this paper). It is indeed interesting that both WapA and WprA are members of the regulon of the YvrGHb TCS, together with the cell-envelope-associated *lytABC* and *dltABCDE* operons (Serizawa *et al.*, 2005). Thus we suggest that the YvrGHb TCS may be involved in adaptation of cell wall metabolism to the phosphate-limited state. The precise functions of WapA and WprA are unknown; ascribing a function to them should yield insight into how their absence facilitates cell envelope adaptation to the phosphate-limited state.

PhoPR function is restricted to the phosphate-limited state

Several new features of the PhoPR-mediated phosphate limitation response emerge from this study. The gene expression profile of cells in the phosphate-limited state differed significantly from that of exponentially growing cells. The relative RNA abundance of ~35 % (1484) and ~43 % (1821) of gene expression changed (increased and decreased in approximately equal numbers) more than twofold when wild-type and AH024 (Δ *phoPR*) strains, respectively, became phosphate limited (Supplementary Tables S5, S6, available with the online version of this paper). Of the gene cohort with altered expression, the RNA abundance of 1232 genes changed more than twofold in both wild-type and AH024 (Δ *phoPR*) cells. However, importantly there was no significant difference in the relative RNA abundance of any gene when exponentially growing wild-type and AH024 (Δ *phoPR*) cells were compared (data not shown). Thus, although *phoPR* is expressed and the PhoPR proteins are present in exponentially growing cells (Sun *et al.*, 1996; Prágai & Harwood, 2002; Bisicchia *et al.*, 2010) PhoPR function is completely restricted to the phosphate-limited state.

Activation and deactivation of the PhoPR-mediated phosphate limitation response

The very rapid but heterogeneous induction of ten PhoPR-regulated promoters determined by LCA is striking (Fig. 2b and Supplementary Fig. S2). These promoters are induced in the following order: the *tuaA* promoter, the *pstS* promoter, the *glpQ* *phoA* *phoB* and *yfkN* promoters, the *phoD* promoter, the *ykoL* and *yurI* promoters, and finally the *yycOP* promoter (Fig. 2b and Supplementary Fig. S2). It is noteworthy that the *tuaA* promoter has the highest number (seven) of PhoP boxes (six of which conform to the derived consensus), perhaps contributing to its early induction. However, there is not a strict correlation

between the number of PhoP boxes/consensus boxes, the order of gene induction and the level of promoter activity (P_{act}), indicating significant complexity of the PhoPR regulon promoter structure. A second important feature is that maximal P_{act} levels of the PhoPR-controlled promoters are sustained throughout an extended (~5 h) period of phosphate limitation. Such sustained maximal P_{act} values contrast with the fluctuating values observed for the majority of the other cell-wall-associated promoters examined in this study (Fig. 2, Supplementary Figs S1 and S2). Thus, the PhoR stimulus clearly persists throughout this period, maintaining the adaptive response. A third feature is the speed with which PhoPR regulon promoters are deactivated upon addition of phosphate to cultures of phosphate-limited cells (Fig. 2a). The P_{act} of PhoPR regulon genes in wild-type cells is reduced to <5 % maximal activity in the 10–20 min interval after phosphate addition (Fig. 2b). Thus the rapid deactivation of the PhoR kinase is on the timescale of metabolite fluctuations, suggesting that the signal emanates from this source. The study by Shi & Hulett (1999) reporting that a partial phosphate limitation response can be induced by a truncated PhoR kinase located cytoplasmically is consistent with the contention that at least part of its activating signal stems from metabolic processes within the cell. Importantly there is a rapid and significant increase in the P_{act} of cell-wall-associated genes upon addition of phosphate to cultures of phosphate-limited cells. Of particular interest is the observation that both wild-type and AH024 (ΔphoPR) cells show similar increased promoter activities (P_{act}). Together these findings show that the PhoPR response is maintained throughout phosphate limitation, adapting cells to this stress condition and maintaining a lowered growth rate. However, it is not required for cell viability since cell wall gene expression in AH024 (ΔphoPR) cells increases with similar kinetics to similar levels to that in wild-type cells upon phosphate replenishment.

Contribution of PhoPR to the physiology of phosphate-limited cells

The direct and indirect contributions of PhoPR to the physiology of phosphate-limited cells can be gauged by the profile of gene expression changes that occur in its presence (i.e. in wild-type cells) and absence [i.e. in AH024 (ΔphoPR)]. In phosphate-limited wild-type cells, the relative RNA abundance of 149 genes shows significant changes of at least twofold, among which are members of the PhoPR regulon (see next section): transcripts of 115 genes increase and of 34 genes decrease (see Supplementary Tables S7 and S8, available with the online version of this paper). The number of genes with decreased expression is fewer than found by Allenby *et al.* (2005) who reported decreased expression of 69 genes including genes encoding proteins involved in DNA synthesis, transcription and translation. Among genes downregulated in our study are those involved in teichoic acid, biotin, cobalamin and sulphur (see below)

metabolism, but not genes involved in central metabolic processes (Table 1, group B). These differences from the study by Allenby *et al.* (2005) are probably due to variations in strain, media [LPDM utilized in this study contains 53 μM phosphate (Müller *et al.*, 1997) while LPDM medium contained 420 μM phosphate in the study by Allenby *et al.* (2005)] and method of analysis (their transcriptome analysis was performed with macroarrays).

In phosphate-limited AH024 (ΔphoPR) cells, the relative RNA abundance of 437 genes shows significant changes of at least twofold: transcripts of 210 genes increased and of 227 genes decreased (see Supplementary Tables S9 and S10, available with the online version of this paper). Among the cohort of 227 genes whose expression decreased are those encoding proteins involved in the central cellular processes including DNA synthesis, translation, cell division and energy production. Expression of these genes does not change to this extent when wild-type 168 cells are phosphate limited. These expression profiles are consistent with continued growth of wild-type cells, but growth cessation of AH024 (ΔphoPR) cells under phosphate-limiting conditions. Importantly, with the exception of the genes encoding teichoic/teichuronic acid synthesis, cell wall genes do not feature significantly among these gene cohorts, showing that cell wall metabolism continues at an appreciable rate in both wild-type and AH024 (ΔphoPR) cells, consistent with the observations of Bisicchia *et al.* (2007). Thus the consequence of PhoPR-mediated adaptation to phosphate-limiting conditions is to maintain gene expression profiles similar to growing and dividing cells in agreement with the observations reported (Bisicchia *et al.*, 2010). However, the PhoPR response is not required to maintain cell viability since AH024 (ΔphoPR) cells resume growth rapidly even after an extended period of phosphate limitation.

The PhoPR regulon

The combined transcriptome (using a tiled array) and LCA analysis allowed refinement of the constituent members of the PhoPR regulon and their relative induction levels upon phosphate limitation. A comparison of the transcriptomes of phosphate-limited wild-type and AH024 (ΔphoPR) cells showed that the RNA abundance of 36 genes increases at least fivefold and that of five genes decreases at least fivefold (Table 1, groups A and B) in wild-type cells only. This gene cohort composes the potential PhoPR regulon. Of the upregulated genes, 27 (Table 1, group A, the top 26 genes excluding *nasD* but including *phoPR*, which is not included in this table as it is induced only approximately threefold upon phosphate limitation) were previously identified as members of the PhoPR regulon (Hulett, 2002; Prágai & Harwood, 2002; Allenby *et al.*, 2005). Although the P_{act} of the *yycOP* operon promoter is unusually low (59 units) for a PhoPR-activated gene (Fig. 2b), transcriptome analysis shows a 26-fold (*yycO*) and a 13-fold (*yycP*) induction

upon phosphate limitation in a PhoPR-dependent manner while Allenby *et al.* (2005) reported a 3.6-fold induction of *yycO* in a wild-type strain and a 15-fold induction in a SigB mutant strain. Together these results are consistent with the *yycOP* operon being a bona fide member of the PhoPR regulon. Among the remaining genes in Table 1, group A, we confirm that *yjdB*, *yfkN* and *yurI* are members of the PhoPR regulon but do not confirm that *yttP*, *vpr*, *yhaX* or *yhbH* are members of this regulon as reported by Prágai & Harwood (2002) and Allenby *et al.* (2005). Moreover, in this study *trePAR* and *yxiO* operons are also potential PhoPR regulon members by virtue of having potential PhoP boxes in their respective promoters. The increased abundance of *nasD* transcripts is probably mediated indirectly through PhoP–P activation of *resDE* expression (Table 1, group A; Supplementary Table S7; Birkey *et al.*, 1998), a view supported by the increased transcript abundance of other ResDE regulon genes (*nasE*, *fnr*, *ctaO* and *ctaD*). Our study also reveals the *yfkN* promoter to be unique among PhoPR regulon members in being active at a low level (P_{act} 64 units) in the absence of phosphate stress (Fig. 2a). Importantly, the high and sustained activities of PhoPR regulon promoters upon induction (Fig. 2b) are consistent with the high induction ratios of PhoPR regulon genes revealed by the transcriptome analysis.

The PhoPR response plays a central role in cell wall anionic polymer metabolism upon phosphate limitation. Our results (Fig. 2b) confirm that promoter activity of the teichuronic acid synthetic operon (*tuaABCDEFHG*) increases very significantly while promoter activity of *tagAB* decreases to $\leq 10\%$ maximal activity in agreement with previous studies (Liu *et al.*, 1998; Lahooti & Harwood, 1999). Interestingly, the promoter activity of the *tagDEF* operon is maintained at up to 25% and that of the *tagGH* operon occurs sporadically at up to 15% of their maximal values during phosphate limitation. The relative RNA abundance of the *tuaABCDEFHG* and *tagAB* operons is consistent with these promoter activities. Moreover the relative abundance of *tagDEF* and *tagGH* transcripts does not change appreciably in phosphate-limited cells (Fig. 2b), consistent with the continued activity of their promoters and strongly suggesting that teichoic acid synthesis occurs in phosphate-limited cells. To address this question we examined the morphology of cells incapable of synthesizing teichuronic acid (complete deletion of the *tuaABCDEFHG*) in the phosphate-limited state. These cells grew normally, entered the phosphate-limited state at the same time as wild-type cells and remained rod-shaped and were indistinguishable from wild-type cells throughout phosphate limitation (data not shown). In contrast, cells depleted for teichoic acids were spherical (data not shown), as previously reported (D'Elia *et al.*, 2006). These observations support our expression analysis (transcriptome and LCA) showing that teichoic acid continues to be synthesized in phosphate-limited cells to an extent sufficient to maintain the normal shape of the bacterium.

Sulphur metabolism in the phosphate-limited state

A novel feature of our transcriptome analysis is the perturbation of sulphur metabolism that occurs in both wild-type and AH024 (Δ *phoPR*) cells in response to phosphate limitation. Transcript abundance of two operons is significantly reduced in wild-type cells upon phosphate limitation (Table 1, group B): there is an approximately eightfold reduction in the *tcyP* (*yhcL*) operon transcript, which encodes an L-cystine transporter (Burguière *et al.*, 2004), and an approximately three- to fivefold reduction in the [(*cysH*) *cysP* *sat* *cysC* *ylnD* *ylnE* *ylnF*] operon transcript, which encodes enzymes involved in sulphate uptake and in its conversion to sulphide (Mansilla & de Mendoza, 2000; Mansilla *et al.*, 2000). The promoters of these operons do not contain potential PhoP boxes, suggesting that regulation is indirect. In addition, expression of several sulphur-metabolism-associated operons is also altered when AH024 (Δ *phoPR*) cells become phosphate limited (Table 1, groups C and D): transcript levels of the *yrrT*, *mccA* and *mccB* genes, which encode enzymes involved in conversion of methionine to cysteine, are increased ~ 13 -fold (Hullo *et al.*, 2007) while transcripts of the *cysIJ* operon (CysI encodes a sulphite reductase) are decreased ~ 16 -fold. The rationale of these gene expression changes under phosphate-limiting conditions is not entirely clear. However, they are indicative of a perturbation in sulphur metabolism that merits further investigation.

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