

## Transcriptome Analysis of Phosphate Starvation Response in *Escherichia coli*

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**Abstract** *Escherichia coli* has a PhoR-PhoB two-component regulatory system to detect and respond to the changes of environmental phosphate concentration. For the *E. coli* W3110 strain growing under phosphate-limiting condition, the changes of global gene expression levels were investigated by using DNA microarray analysis. The expression levels of some genes that are involved in phosphate metabolism were increased as phosphate became limited, whereas those of the genes involved in ribosomal protein or amino acid metabolism were decreased, owing to the stationary phase response. The upregulated genes could be divided into temporarily and permanently inducible genes by phosphate starvation. At the peak point showing the highest expression levels of the *phoB* and *phoR* genes under phosphate-limiting condition, the *phoB*- and/or *phoR*-dependent regulatory mechanisms were investigated in detail by comparing the gene expression levels among the wild-type and *phoB* and/or *phoR* mutant strains. Overall, the *phoB* mutation was epistatic over the *phoR* mutation. It was found that PhoBR and PhoB were responsible for the upregulation of the phosphonate or glycerol phosphate metabolism and high-affinity phosphate transport system, respectively. These results show the complex regulation by the PhoR-PhoB two-component regulatory system in *E. coli*.

**Key words:** Phosphate starvation response, PhoR-PhoB two-component regulatory system, *Escherichia coli*, DNA microarray, transcriptome

Phosphorus is an essential element of various biomolecules, and has an important role in signal transfer pathways [17].

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Moreover, phosphorus metabolism is closely related with diverse metabolisms including energy and central carbon metabolisms [8]. For this reason, the cellular physiology affected by phosphorus metabolism is quite complex. All living cells sophisticatedly regulate the mechanisms of phosphate uptake and assimilation to adapt and survive in phosphate-limiting condition [1, 18].

Depending on the concentration of environmental phosphate, *Escherichia coli* controls phosphate metabolism through a PhoR-PhoB two-component regulatory system. In the PhoR-PhoB two-component regulatory system, PhoR senses the environmental phosphate concentration as a sensor kinase, and PhoB responds to phosphate starvation as a response regulator or transcriptional activator [17]. Additionally, it has been reported that the phosphate starvation response by this PhoR-PhoB two-component regulatory system is affected by other metabolic pathways, and affects the gene regulations involved in other metabolisms [1].

In order to understand this complex regulation, a systems level analysis of cellular responses to environmental phosphate limitation is required. Even though the phosphate starvation response in *E. coli* has been studied in depth at the molecular and proteome levels [16, 17], a transcriptome level analysis has not yet been performed [9]. In our previous study [1], novel gene members in the Pho regulon were identified based on transcriptome analysis. Those genes are not directly related with phosphate metabolism, suggesting additional roles of PhoB as a global regulator. In this study, the changes in global gene expression levels during the growth of *E. coli* under phosphate-limiting condition were analyzed using DNA microarray. Then, the genes subject to *phoB*- and/or *phoR*-dependent or -independent regulation were identified and classified by comparing the transcriptome profiles of *phoB* and/or *phoR* knockout mutants with that of wild-type in the response to phosphate starvation.

**Table 1.** Strains and plasmids used in this study.

Strain or plasmid	Relevant characteristic(s) <sup>a</sup>	Reference or source
<i>E. coli</i> strains		
W3110	Derived from K-12, $\lambda^-$ , F <sup>-</sup> , prototrophic	KCTC <sup>b</sup> 2223
WB	W3110 <i>phoB</i> ::Km <sup>r</sup>	[1]
WR	W3110 <i>phoR</i> ::Km <sup>r</sup>	This study
WBR	W3110 <i>phoBR</i> ::Km <sup>r</sup>	This study
Plasmids		
pKD46	Red recombinase expression vector, temperature-sensitive <i>ori</i> , Ap <sup>r</sup>	[2]
pACYC177	Cloning vector, Ap <sup>r</sup> , Km <sup>r</sup>	New England Biolabs <sup>c</sup>

<sup>a</sup>Abbreviations: Ap<sup>r</sup>, ampicillin resistance; Km<sup>r</sup>, kanamycin resistance.

<sup>b</sup>Korean Collection for Type Cultures, Daejeon, Korea.

<sup>c</sup>New England Biolabs, Beverly, MA, U.S.A.

## MATERIALS AND METHODS

### Bacterial Strains and Plasmids

The bacterial strains and plasmids used in this study are listed in Table 1. For the construction of the mutant strains (WR and WBR), the *phoR* and *phoBR* genes were disrupted by the homologous recombination system using  $\lambda$  Red recombinase [1, 2]. The oligonucleotides used for gene deletions are listed in Table 2.

### Cultivation

For the general gene manipulation, *E. coli* was cultivated in Luria-Bertani (LB) medium (10 g/l tryptone, 5 g/l yeast extract, and 5 g/l NaCl). If required, ampicillin (50  $\mu$ g/ml) and/or kanamycin (35  $\mu$ g/ml) were supplemented. For phosphate starvation experiments, cells were cultivated as described previously [1]. Briefly, cells were cultivated in 3-(N-morpholino)propanesulfonic acid (MOPS) minimal medium [12] containing 4 g/l glucose. To make P-limiting and -sufficient conditions, 0.2 and 1 mM K<sub>2</sub>HPO<sub>4</sub>, respectively, were also added. Cells were grown at 37°C with shaking at 200 rpm unless otherwise specified.

### DNA Microarray Analysis

All procedures including RNA preparation, cDNA labeling, DNA hybridization, and data analysis were carried out as described previously [1]. GenePloer TwinChip *E. coli*-6 K

oligo chips (GT3001, Digital Genomics, Korea) were used according to the manufacturer's protocol. The microarray images were obtained using the Axon Scanner (Axon Inc., Union City, CA, U.S.A.), and analyzed using the GenePix 3.0 (Axon Inc.) and Genesis 1.5.0. beta 1 (<http://genome.tugraz.at>) softwares.

### Other Measurements

Cell concentration was determined by measuring the OD<sub>600</sub> on a spectrophotometer (Ultraspec3000, Pharmacia Biotech, Sweden). The concentration of phosphate was determined colorimetrically by using the ascorbic acid method [3].

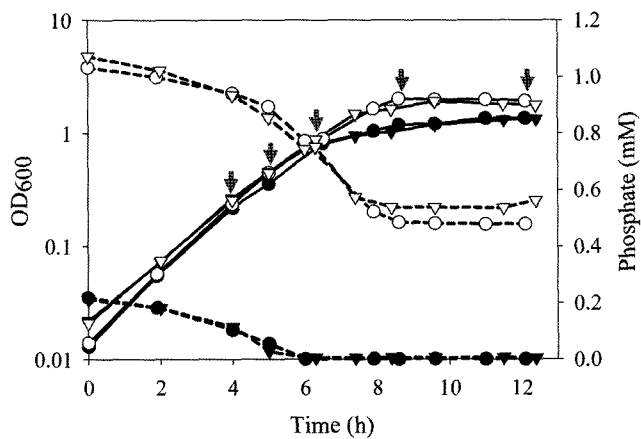
## RESULTS AND DISCUSSION

### Transcriptome Profiling of *E. coli* W3110 During the Growth Under P-Limiting Condition

The growth profiles of W3110 and its mutant strains (WB, WR, and WBR) were similar. The growth profiles of WR and WBR in this study resembled those of W3110 and WB in the previous study [1]. This means that the deletions of the *phoB* and/or *phoR* genes did not affect the growth significantly in this culture condition. It can be said that all of the strains entered the stationary phase at the time point of phosphate exhaustion (6.3 h) under P-limiting condition, as shown in Fig. 1. Total RNAs were prepared from the

**Table 2.** Oligonucleotides used in this study.

Target	Aim	Primer	Sequence (5'-3')
<i>phoBR</i>	1st PCR for deletion	PhoB-F1	AATCAATAACCTGAAGATATGTGCGTTAAGTATCTTCCTGGCATC
		PhoR-R1	GGCAATAAAAAGATGACAAAGGCGGAGTAATGCTCTGCCAGTGT
	2nd PCR for deletion	PhoB-F2	CATTTGCTTTTTTCTGCGCCACGGAAATCAATAACCTGAAGATATGT
		PhoR-R2	AATAAGCCAGCATCGACTGGCTTATGGCAATAAAAAGATGA CAAAGG
<i>phoR</i>	1st PCR for deletion	PhoR-F1	CAGAGCAGGGCTTATGATTTCTTAATTAAGTATCTTCCTGGCATC
		PhoR-R1	GGCAATAAAAAGATGACAAAGGCGGAGTAATGCTCTGCCAGTGT
	2nd PCR for deletion	PhoR-F2	CTTTTAACGCCTTGCTCATCGGACGCAGAGCAGGGCTTATGATT
		PhoR-R2	AATAAGCCAGCATCGACTGGCTTATGGCAATAAAAAGATGACAAAGG



**Fig. 1.** Cultivation of *phoR* and *phoBR* mutant strains under P-limiting and P-sufficient conditions.

Time profiles of the concentrations of cells (solid line;  $\circ$  and  $\nabla$  are for WR and WBR, respectively) and phosphate (dashed line) during the growth under P-limiting (solid symbols) and P-sufficient (open symbols) conditions. Arrows indicate the sampling time (4.2, 5.2, 6.3, 8.8, and 12 h) for transcriptome analysis.

cells at the  $OD_{600}$  of 0.3 (4.2 h), 0.6 (5.2 h), 0.9 (6.3 h), 1.2 (8.8 h), and 1.5 (12 h), and were used for transcriptome analysis as mentioned in the previous study [1]. Transcriptome levels obtained from the samples at the  $OD_{600}$  of 0.6, 0.9, 1.2, and 1.5 were compared with that at the  $OD_{600}$  of 0.3 using DNA microarrays. The numbers of genes showing significantly different expression levels ( $p$ -value  $\leq 0.05$ ) were 39, 43, 47, and 49 upregulated genes, and 11, 7, 3, and 1 downregulated genes at the  $OD_{600}$  of 0.6, 0.9, 1.2, and 1.5, respectively (Table 3). This means that the expression of many genes was activated rather than repressed as cells grew and/or phosphate became limited. This is consistent with the reported finding that the phosphorylated PhoB only activates the transcription of the related genes, but does not repress gene transcription under P-limiting condition [17]. The genes that were expressed differentially by more than 2-fold in at least one comparison were selected (1,334 out of 3,994 genes). These selected genes could be grouped into 9 clusters through 50 maximum iterations by K-means clustering (Fig. 2).

**Table 3.** Changes of selected gene expression levels during the growth of *E. coli* W3110 under P-limiting condition.

Symbol	Gene <sup>a</sup> Description	Log <sub>2</sub> ratio of gene expression (based on $OD_{600}$ of 0.3)			
		$OD_{600}$			
		0.6	0.9	1.2	1.5
amn	AMP nucleosidase	0.205828	1.649952	2.173173	0.757537
araH_2	High-affinity L-arabinose transport system; membrane protein	1.145471	2.969035	0.961001	1.031313
b0379	orf	0.113032	3.934411	1.663268	1.541482
b1499	Putative ARAC-type regulatory protein	-0.42197	0.162178	2.030278	1.127551
b1593	orf	1.766969	1.007289	0.371711	0.844478
b1788	orf	0.682693	0.66266	2.219955	1.259717
b1973	orf	4.673794	0.411525	4.482142	2.2473
b3027	orf	0.069867	0.451407	2.119297	1.377464
b4103	orf	0.639835	2.409552	2.716467	1.267133
creC	Catabolite repression sensor kinase for PhoB; alternative sensor for the Pho regulon	0.297902	0.396201	-0.42176	0.228438
dmsB	Anaerobic dimethyl sulfoxide reductase subunit B	1.697784	0.534722	0.976642	0.918378
frdA	Fumarate reductase	1.706047	0.816457	0.741136	0.88201
fruK	Fructose-1-phosphate kinase	1.893047	0.161445	0.117484	0.740261
gadA	Glutamate decarboxylase isozyme	1.211624	2.854647	3.14422	0.564976
gadB	Glutamate decarboxylase isozyme	1.020541	2.747133	2.545004	0.446747
hisD	L-Histidinal:NAD <sup>+</sup> oxidoreductase; L-histidinol:NAD <sup>+</sup> oxidoreductase	1.627586	-0.44307	-0.91542	-0.23295
hycD	Membrane-spanning protein of hydrogenase 3 (part of FHL complex)	-0.41008	0.581915	2.084467	1.067389
metJ	Repressor of all <i>met</i> genes except <i>metF</i>	0.976838	2.876008	-0.44632	0.692011
mhpA	3-(3-Hydroxyphenyl)propionate hydroxylase	0.55739	6.799518	1.555454	1.765856
pgsA	Phosphatidylglycerophosphate synthetase = CDP-1	0.479647	1.387668	1.92756	1.34969
phnC	ATP-binding component of phosphonate transport	1.180111	3.00424	4.156415	2.46907
phnD	Periplasmic binding protein component of Pn transporter	2.480893	4.857272	4.377642	3.98894
phnJ	Phosphonate metabolism	0.156094	1.496322	2.144232	1.351872
phoA	Alkaline phosphatase	2.601825	3.126163	3.145885	1.878072
psiF	Induced by phosphate starvation	1.478743	2.428061	2.011654	1.364459

Table 3. Continued.

Gene <sup>a</sup>		Log <sub>2</sub> ratio of gene expression (based on OD <sub>600</sub> of 0.3)			
Symbol	Description	OD <sub>600</sub>			
		0.6	0.9	1.2	1.5
<i>rhsC</i>	<i>rhsC</i> protein in <i>rhs</i> element	2.02699	2.739195	2.182582	1.727511
<i>sbmA</i>	Sensitivity to microcin B17	0.286825	0.95767	2.024715	2.171023
<i>trkG</i>	<i>trk</i> System potassium uptake; part of Rac prophage	0.02558	1.059229	2.150764	1.343959
<i>ugpB</i>	sn-Glycerol 3-phosphate transport system; periplasmic binding protein	1.595289	2.064705	2.895675	1.380991
<i>xasA</i>	Acid sensitivity protein	-0.07552	0.624097	2.632345	0.579373
<i>yagP</i>	Putative transcriptional regulator LYSR-type	-0.5089	-0.19662	2.066086	1.534636
<i>yaiT</i>	orf	0.621609	3.143466	1.493996	1.716672
<i>ybfB</i>	orf	0.357186	0.698934	2.461438	0.944435
<i>ycaC</i>	orf	0.783761	2.027743	2.363813	1.245716
<i>ycgK</i>	orf	1.561187	1.951167	1.438053	0.548108
<i>ycgW</i>	orf	0.060575	0.201867	2.422763	0.971501
<i>yebL</i>	Putative adhesin	1.580117	1.552778	1.390501	0.936959
<i>yedN</i>	orf	0.449752	0.682785	2.041299	1.28143
<i>yeeD</i>	orf	4.846523	-0.90632	-0.49433	2.271431
<i>ygcG</i>	orf	-0.56718	-0.16126	1.991295	1.01999
<i>yglI</i>	Putative oxidoreductase	-0.30308	0.082769	2.439569	1.07375
<i>yhiE</i>	orf	1.412082	2.100454	2.673947	0.750404
<i>yhiF</i>	orf	-0.12268	-0.00146	4.124502	0.582875
<i>yhiJ</i>	orf	0.206058	0.538691	2.387379	1.365863
<i>yibD</i>	Putative regulator	0.489649	3.254333	3.994242	2.658403
<i>yigE</i>	orf	-0.2226	0.583685	2.007139	1.367115
<i>yjbL</i>	orf	0.645207	3.025273	0.56513	2.960589
<i>yjgN</i>	orf	-0.17657	-0.09218	2.016379	1.15897
<i>yjiO</i>	Putative transport protein	-0.19775	0.520607	2.019336	1.400563
<i>ymfE</i>	orf	-0.13302	-0.83354	2.494803	0.950194
<i>ytjK</i>	orf	0.536059	1.978537	2.736494	1.582685

<sup>a</sup>The genes having the *p*-value of less than 0.05 were selected except for the *creC* gene.

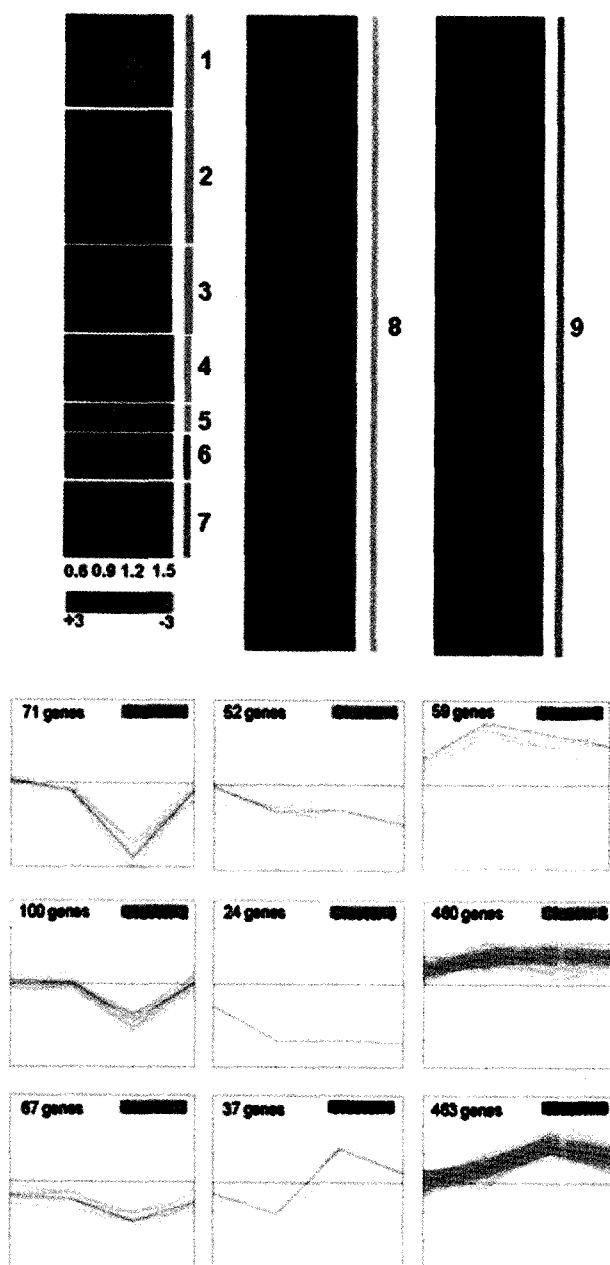
### Stationary Phase Inducible Gene Expression

Clusters 1, 2, and 3 contain the genes (71, 100, and 67 genes, respectively) showing low expression levels at mid-stationary phase (OD<sub>600</sub> of 1.2). These clusters contain the majority of the ribosomal protein genes (*rpl*ACEFJKLMNOQPRSUVWXY and *rpm*ABCDEFGHJI for 50S, and *rps*ABDEFHJKMNPOQSU for 30S, mostly in cluster 1). The genes for purine ribonucleotide biosynthesis (*pur*ACDEGHU, mostly in cluster 3), RNA polymerase (*rpo*ABC), membrane-bound ATP synthase (*atp*ABEF, mostly in cluster 1), galactitol or tagatose uptake metabolism (*gat*ABCYZ, mostly in cluster 1), PTS system (*pts*GHI), and arginine metabolism (*arg*ABDGHI, mostly in cluster 2) were also found in these clusters. There have been several reports showing the repression of genes involved in amino acid metabolism or ribosomal protein in other microorganisms [8, 10] and *E. coli* at the proteome level [16] when phosphate is limited. They concluded that this was consistent with the slower growth rate of cells under P-limitation. It has been generally known that the expression of the amino acid biosynthesis

genes and ribosomal protein genes is repressed at the stationary phase [4, 13]. The genes in these clusters are related to overall growth inhibition caused by various stress conditions at the stationary phase, rather than only by phosphate starvation.

Clusters 4 and 5 contain the genes (52 and 24 genes, respectively) that show decreasing expression levels after the exponential phase (OD<sub>600</sub> of 0.9). In these clusters, the genes encoding the IS 1 protein (*insA*<sub>2</sub>, *insA*<sub>3</sub>, and *insB*<sub>3</sub>) were found. Additionally, the genes for homoserine or threonine metabolism are involved in cluster 5 (*thr*ABC). Cluster 4 contains the genes for isopropylmalate metabolism (*leu*ABC), and chorismate metabolism (*aro*CP and *phe*A). This indicates that some genes involved in amino acid biosynthesis and metabolism were downregulated. As in clusters 1, 2, and 3, this may be due to the general repression at the stationary phase.

Cluster 6 contains 37 genes showing dynamic changes of expression. Their expression decreased during the exponential phase (OD<sub>600</sub> of 0.9), changed to increase and



**Fig. 2.** K-means clustering analysis of the changes of gene expression levels during the growth of *E. coli* W3110 under P-limiting condition.

The expression levels at the  $OD_{600}$  of 0.6 (5.2 h), 0.9 (6.3 h), 1.2 (8.8 h), and 1.5 (12 h) were compared with that at the  $OD_{600}$  of 0.3 (4.2 h). The genes (1,334 genes) that were expressed differentially by more than 2-fold in at least one comparison were selected.

peaked at the mid-stationary phase ( $OD_{600}$  of 1.2), and decreased again afterwards. This cluster includes some genes in the *uhp* operon (*uhpBC*) for hexose-6-phosphate transport. Hexose-6-phosphate can be used as a phosphate source, but there is no evidence that the genes for hexose 6-phosphate assimilation are regulated by PhoB [14]. In addition, citrate-dependent iron transport genes (*fecCD*)

and putative transcriptional regulator genes in cell process (*envR* and *sgcR*) were found in this cluster. Overall, cluster 6 contains some genes involved in phosphate metabolism. However, these genes do not seem to be regulated by PhoB.

### Phosphate Starvation-Dependent Gene Expression

Cluster 7 contains 59 genes showing the peak expression levels at the end of the exponential phase ( $OD_{600}$  of 0.9), the point of phosphate exhaustion. The genes involved in phosphate metabolism and typically induced by phosphate starvation were found in this cluster: *phnCDGJ* for phosphonate uptake and metabolism, *phoA* for alkaline phosphatase, *psiF* for the metabolism of phosphorus compounds, and *ugpB* for glycerol-3-phosphate and glycerolphosphoryl diester uptake. These genes have been regarded as phosphate starvation inducible genes in the other studies [17]. The genes encoding phosphatidylglycerophosphate synthetase or diacyl-sn-glycerol-3-phosphate phosphatidyl transferase (*pgsA*) and putative phosphonmutase-citrate synthase (*prpBC*) were also found in cluster 7. Additionally, the genes involved in global regulation under starvation condition (*dps*), arabinose metabolism (*araA* and *araH\_2*), and glutamate metabolism (*gadAB*) were found in this cluster. Examination of the expression patterns of ribosomal protein genes in clusters 1, 2, and 3 revealed that the upregulation of phosphate starvation inducible genes occurs earlier than the downregulation of ribosomal protein genes. This indicates that the phosphate starvation response was initiated before cell growth slowed down at the stationary phase in the condition of this experiment. Based on these results, it can be said that the regulation of phosphate starvation inducible genes is strictly dependent on environmental phosphate concentration rather than the growth phase itself.

Cluster 8 contains 460 genes showing high expression levels constantly after the exponential phase ( $OD_{600}$  of 0.9), that is, after phosphate exhaustion. In this cluster, many phosphate starvation inducible genes under the regulation by PhoB were found: the genes for phosphonate metabolism (*phnKMN*, and *phnB* moreover), high-affinity phosphate transport system (*pstAS*), and the Pho regulatory function (*phoBHRU*). This implies that cells activate phosphate uptake and metabolism systems under the regulation by PhoB to scavenge and utilize environmental phosphate and phosphorus compounds.

Cluster 9 contains 463 genes, the expression levels of which peaked at the mid-stationary phase ( $OD_{600}$  of 1.2). The genes for sn-glycerol-3-phosphate and glycerolphosphoryl diester uptake (*ugpAE*), and phosphonate metabolism (*phnFH*) were found in this cluster. Interestingly, many genes in xylose metabolism (*xyxABEFGH*) belong to this cluster [14]. Additionally, the genes for the low-affinity phosphate transport system (*pitB*) and protein phosphatase (*pphB*) were found. In contrast to the genes of the high-affinity phosphate

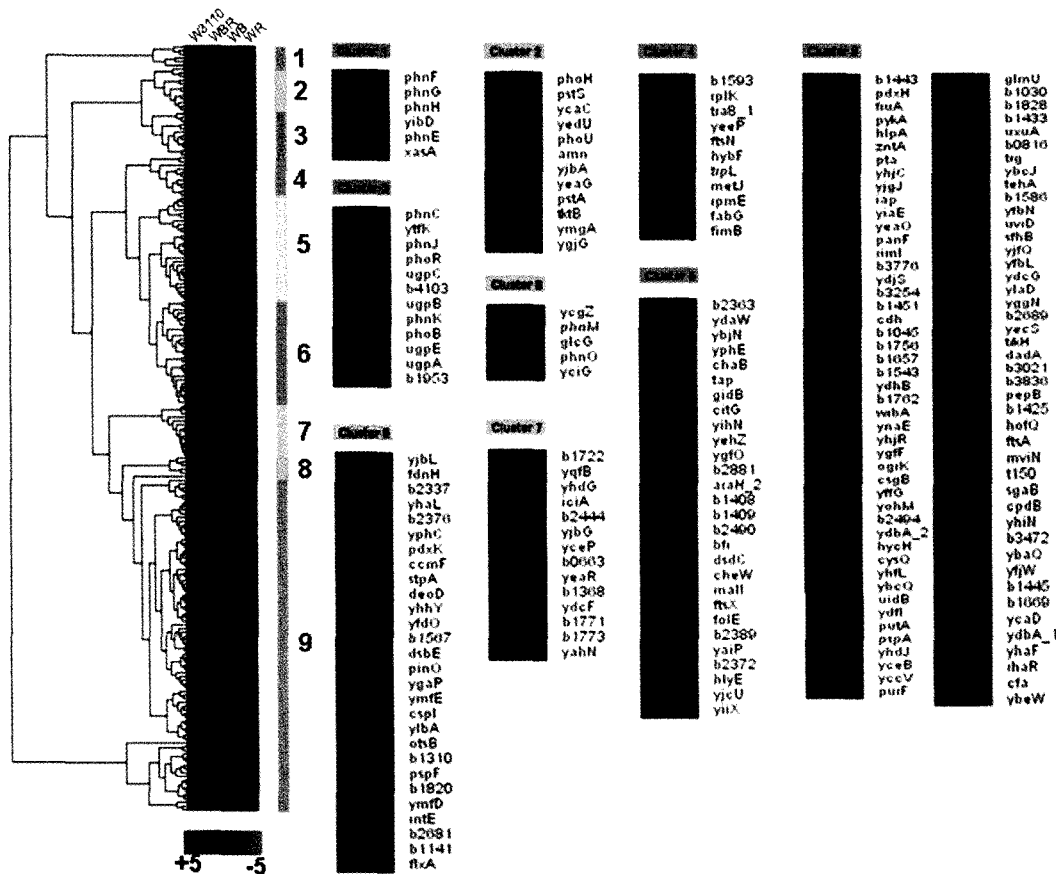
transport system (*pstAS*) found in cluster 8, the gene of the low-affinity phosphate transport system (*pitB*) found in cluster 9 was expressed constitutively and not regulated by PhoB as previously known [17]. This means that some phosphate starvation response genes are not so sensitive to phosphate concentration. On the other hand, the *pstAS* genes, which belong to cluster 8, were strictly dependent on phosphate concentration.

**Identification and Classification of the Genes Under *phoB* and/or *phoR* Regulation**

Based on the results of DNA microarray experiments on the phosphate starvation responses of *E. coli* W3110 strain, we next examined in further detail the regulatory controls exerted by PhoB and/or PhoR [1]. Three knockout strains WB, WR and WBR, which lack the *phoB*, *phoR*, and *phoBR* genes, respectively, were used. As mentioned above, the growth of WB strain was similar to that of wild-type strain, and there was little difference in growth profiles under the current experimental P-limiting and -sufficient conditions [1]. The growth profiles of WR and WBR strains were also similar to those of wild-type and WB

strains (Fig. 1). This allows suitable transcriptome analysis by minimizing the effect of growth inhibition itself.

As explained in the previous study [1], the samplings for transcriptome profiling were carried out at the time between the exponential and stationary phases, just after phosphate was completely exhausted (6.3 h). At this time point, the expression levels of the *phoB* and *phoR* genes were highest [1]. After that, their expression levels were decreased during the stationary phase, suggesting the high stability of PhoB and PhoR [1, 6, 7]. Total RNAs were prepared from 4 strains under 2 different conditions: W3110, WB, WR, and WBR in P-limiting and -sufficient conditions. The global gene expression profiles under P-limiting condition were compared with those under P-sufficient condition for each of the strains [1]. The *phoB* and *phoR* genes were highly expressed in response to phosphate starvation in the wild-type strain, as expected. On the other hand, the expression levels of the *phoR* gene in WB and the *phoB* gene in WR were less than the typical levels under P-limiting condition since they were required for cross-activation [17]. The 204 genes (out of 3,361 genes) showing differential expression (based on the *p*-value of less than 0.05 in at least one



**Fig. 3.** Hierarchical clustering analysis of the changes of gene expression levels in W3110, WB, WR, and WBR under P-limiting and P-sufficient conditions. The genes (204 genes) that were expressed differentially (*p*-value of less than 0.05) in at least one comparison were selected.

strain) were selected (Supplementary Table 1). They were classified by hierarchical clustering (Fig. 3), and analyzed in detail.

#### ***phoB* and/or *phoR*-Dependent Upregulation**

Clusters 1 and 3 contain the genes (6 and 12 genes, respectively) showing *phoB*- and *phoR*-dependent upregulation in response to phosphate starvation. In these clusters, the genes related to phosphonate metabolism (*phnEFGH* more remarkably, and *phnCJK*), and the glycerol-3-phosphate transport system (*ugpABCE*) were found, as expected. Additionally, a gene of unknown function, b4103, which is located in the *phn* operon, was found. Unexpectedly, the expression of the *yibD* (putative regulator), *yfK* (hypothetical protein), and *xasA* (acid sensitivity protein, putative transporter) genes was found to be activated by PhoB and PhoR. Among them, the *xasA* gene was highly expressed in *E. coli* W3110 at the mid-stationary phase, as described earlier (Table 3, belonging to cluster 9 in Fig. 2). It could be that acid stress related genes are highly expressed as the culture medium becomes more acidic in the stationary phase. As found in this study, some of the genes such as *xasA* seem to be additionally activated indirectly by PhoB regulation when phosphate is limited [1]. Similarly, Suziedeliene *et al.* [15] reported that the expression of the *asr* gene, which is involved in acid shock response, was regulated by PhoB in *E. coli*. These results suggest that phosphate starvation and acid stress responses affect each other.

Clusters 2 and 8 contain 12 and 5 genes, respectively, that are upregulated in a *phoB*-dependent but *phoR*-independent manner. The genes for high-affinity phosphate transport (*pstAS*), Pho regulon control (*phoHU*), and phosphonate metabolism (*phnMO*) were found in these clusters. These genes are expected phosphate starvation inducible genes and show high expression after the exponential phase (phosphate exhaustion) in the study on the growth-associated response to phosphate starvation (Table 3, belonging to clusters 7 and 8 in Fig. 2). Therefore, it can be suggested that these gene are *phoB*-dependent and phosphate starvation inducible.

In contrast, some genes such as *glcG* (hypothetical protein) and *yjbA* (hypothetical protein) show the same expression patterns but have no putative Pho box. Although it is suggested that the expression of the *yjbA* gene is under the regulation by PhoB (<http://regulondb.ccg.unam.mx/index.html>), the Pho box consensus sequence was not found. Nonetheless, it is hasty to conclude that these genes are not under regulation by PhoB. This is because there are unusual cases of DNA-protein interaction without a consensus sequence, like Ada (regulator protein) binding to the promoter region of the *aidB* gene (regulated gene) [11].

It could be confirmed that the *phoB*- and/or *phoR*-dependent upregulated genes found in clusters 1, 2, 3, or 8

in Fig. 3 were highly expressed in the exponential and/or stationary phases (after phosphate exhaustion) during the study on the growth-associated response to phosphate starvation (belonging to clusters 7, 8, or 9 in Fig. 2). It was also observed that the *phoB* mutation is epistatic to the *phoR* mutation as the WBR strain showed more similar overall differences of gene expression patterns to the WB strain than the WR strain.

#### **Other *phoB* and/or *phoR*-Dependent Gene Regulations**

Clusters 4 and 6 contain the genes (11 and 28 genes, respectively) showing relatively high expression levels in WB in response to phosphate starvation. The genes involved in cell process (*cheW*, *fisNX*, and *tap*) and translation (*araH\_2*, *bfr*, *rplK*, and *rpmE*) were found in these clusters. These genes were downregulated in W3110 at the stationary phase under P-limiting condition (belonging to clusters 1, 2, and 3 in Fig. 2). It was thought that these genes were not regulated by PhoB during the transcriptome analysis of the W3110 strain (Fig. 2). However, the expression levels of these genes were changed depending on the existence of the *phoB* and *phoR* genes. The genes found in cluster 4 can be interpreted as the *phoB*- and *phoR*-dependent downregulated genes. More generally speaking, the genes in these clusters can be said to be regulated by PhoB indirectly through complex regulatory networks. For example, the expression level of the *araH\_2* gene increased during the growth under P-limiting condition (Table 3). However, the difference of its expression levels in the W3110 strain under P-limiting and -sufficient conditions was small. Furthermore, its expression level was different depending on the presence of the *phoB* and/or *phoR* genes (Supplementary Table 1). This means that this gene is a stationary phase inducible gene that is indirectly regulated by PhoB.

Cluster 5 contains 28 genes showing upregulation in mutant strains in response to phosphate starvation. Mostly function-unknown genes and some genes involved in energy metabolism (*ccmF*, *dsdE*, and *fdnH*) were found in this cluster. These genes showed higher expression levels when *phoB* and/or *phoR* expression was lower. It has not been reported that the expression of a gene in *E. coli* or other microorganisms is repressed depending on the *phoB* and/or *phoR* expression levels. Even though it is not right to say that downregulation of some, if not all, of these genes are truly regulated by PhoB, the expression of these genes is directly or indirectly linked with *phoBR* levels. Only weak homologies to the proposed consensus sequence of the Pho box were observed in the genes of this cluster [1]. Further study is needed to understand the relationship between the PhoBR and the expression of the genes in this cluster.

Cluster 7 contains 14 genes showing upregulation in response to phosphate starvation when the *phoR* gene was disrupted. All genes in this cluster are of unknown function except the *iciA* (replication initiation inhibitor) gene. Han

*et al.* [5] reported that the expression of the *iciA* gene was activated depending on PhoB under phosphate starvation. In this study, the expression of the genes found in cluster 7 might be repressed directly or indirectly by PhoR, but not by PhoB, since the expression levels were similar between W3110 and WB, and WBR and WR, respectively. This means that the effect of PhoR is stronger than PhoB on the expression of the genes in this cluster. Furthermore, their expression seems to be independent of phosphate concentration since there were no differences in the gene expression levels between P-limiting and -sufficient conditions in W3110. The genes in this cluster show upregulation in the absence of the *phoR* gene. This does not truly mean that their expression is repressed by PhoR. If PhoR does not act as a direct transcription repressor, it may trigger other transcription factors for the repression of these genes.

Cluster 9 has 91 genes showing upregulation only when the *phoR* gene was disrupted in response to phosphate starvation. The genes involved in cell process (*tehA*, *trg*, *fts*, and *mvnN*), and transport and binding (*hofQ*, *panF*, *trkH*, and *uidB*) were found in this cluster. The genes involved in phosphorus-related molecule metabolism such as the *iap* (alkaline phosphatase isozyme conversion), *pta* (phosphate acetyltransferase), *cdh* (CDP-diacylglycerol phosphotidylhydrolase), and *cfa* (cyclopropane fatty acyl phospholipid synthase) genes were also present. The role of PhoR in maintaining the expression levels of these genes in response to phosphate starvation seems to be important since the expression levels were highly increased in WR.

### Cross-Regulation by CreC

CreC (catabolite repression sensor kinase, alternative sensor kinase for the Pho regulon) activates the *phoB* gene expression as a cross-regulator and interconnects the central pathways of carbon and energy metabolism [17]. The log<sub>2</sub> ratios of the changed expression levels of the *creC* gene were 0.297902, 0.396201, -0.42176, and 0.228438 at the OD<sub>600</sub> of 0.6, 0.9, 1.2, and 1.5 based on the OD<sub>600</sub> of 0.3, respectively, in W3110 during the growth under P-limiting condition (Table 3). This result is supported by the previous finding that the *creC* gene is expressed constitutively independent of phosphate concentration when using glucose as a carbon source [17]. It is also possible that the expression level of the *creC* gene was already high at the initial sampling time (at OD<sub>600</sub> of 0.3) because this gene was promptly upregulated before phosphate exhaustion. The log<sub>2</sub> ratio of the different expression levels of the *creC* gene under P-limiting and -sufficient conditions were 0.266169, -0.62488, 2.054329, and 1.460942 for W3110, WB, WR, and WBR, respectively (Supplementary Table 1). The similar expression level of the *creC* gene in W3110 also suggested that the expression of this gene is independent of phosphate concentration. The expression level of the *creC* gene

significantly increased under P-limiting condition when the *phoR* gene was deleted. This suggests that CreC plays a role as an alternative sensor kinase instead of PhoR in response to phosphate starvation. This shows the link connecting carbon and phosphate metabolisms, and it is a way to fine control the cellular metabolisms.

In conclusion, the expression levels of phosphate starvation response genes were different depending on the time and the existence of PhoB and/or PhoR. Additional roles of the PhoR-PhoB two-component regulatory system could be suggested as follows. First, we were able to identify and classify the genes that are regulated positively or negatively in a *phoB*- and/or *phoR*-dependent manner. The results of transcriptome analysis of W3110 and its *phoB* and/or *phoR* mutant strains suggested the possible role of PhoR itself as a transcriptional regulator. Interestingly, PhoB-dependent downregulation of the expression of some genes was observed. This is most likely due to the interaction of the PhoR-PhoB two-component regulatory system with other regulatory circuits and metabolisms, which deserves further study. The expression of the genes under the control of the PhoR-PhoB two-component system in *E. coli* was found to be affected by the duration of P-limitation, in response to phosphate starvation. This means that the roles of the PhoR-PhoB two-component regulatory system in overall *E. coli* metabolism under P-limiting condition seem to be more complex and highly interconnected with other regulatory circuits than previously known.

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