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Reciprocal Regulation of GlnR and PhoP in Response to Nitrogen and Phosphate Limitations in *Saccharopolyspora erythraea*

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Nitrogen and phosphate source sensing, uptake, and assimilation are essential for the growth and development of microorganisms. In this study, we demonstrated that SACE_6965 encodes the phosphate regulator PhoP, which controls the transcription of genes involved in phosphate metabolism in the erythromycin-producing *Saccharopolyspora erythraea*. We found that PhoP and the nitrogen regulator GlnR both regulate the transcription of *glnR* as well as other nitrogen metabolism-related genes. Interestingly, both GlnR- and PhoP-binding sites were identified in the *phoP* promoter region. Unlike the nonreciprocal regulation of GlnR and PhoP observed in *Streptomyces coelicolor* and *Streptomyces lividans*, GlnR negatively controls the transcription of the *phoP* gene in *S. erythraea*. This suggests that GlnR directly affects phosphate metabolism and demonstrates that the cross talk between GlnR and PhoP is reciprocal. Although GlnR and PhoP sites in the *glnR* and *phoP* promoter regions are located in close proximity to one another (separated by only 2 to 4 bp), the binding of both regulators to their respective region was independent and noninterfering. These results indicate that two regulators could separately bind to their respective binding sites and control nitrogen and phosphate metabolism in response to environmental changes. The reciprocal cross talk observed between GlnR and PhoP serves as a foundation for understanding the regulation of complex primary and secondary metabolism in antibiotic-producing actinomycetes.

Soil-dwelling actinomycetes produce many valuable secondary metabolites that exhibit important pharmacological characteristics and have uses as antibiotics, immunosuppressants, anticancer agents, and many other bioactive compounds. Biosynthesis of these secondary metabolites involves complex regulatory interaction networks, which are themselves finely tuned by many intra- and extracellular signals associated with physicochemical stress and the availability of carbon, nitrogen, and phosphate sources (1–10). Concentrations of easily utilizable nitrogen and phosphate sources in the fermentation medium are critical for secondary-metabolite production (11–17).

Nitrogen control of metabolism in several *Actinomycetes* species is mediated by a response regulator, GlnR, belonging to the OmpR family (10, 18). The GlnR-mediated transcription regulatory network that coordinates the control of expression of genes involved in nitrogen assimilation, nitrogen metabolism, and other metabolic activity has been explored in *Streptomyces coelicolor* (19, 20) and *Streptomyces venezuelae* (21). PhoP is a response regulator of the phosphate-sensing two-component system PhoP-PhoR and has been widely investigated in *S. coelicolor* and *Streptomyces lividans* (12, 22–24). Recently, it was proposed that PhoP plays a central role in primary and secondary metabolism in *S. coelicolor* (25). A complete nutrient-sensing signal transduction pathway, PhoP-AfsR-AfsS-SARP (where SARP represents a *Streptomyces* antibiotic-related protein, such as ActII-orf4 or RedD), was elucidated to demonstrate that the phosphate-regulatory effect on secondary metabolism was exerted via a cross talk between PhoP and the AfsR and AfsS regulators. The studies also observed that the expression of the *glnR* gene and some other GlnR-regulated genes is repressed by PhoP in *S. coelicolor* (5, 24–27). PhoP repressed the transcription of these nitrogen genes by its binding to the *glnR* promoter, encoding the major nitrogen regulator, to the promoters of *glnA* and *glnII*, encoding two glutamine synthetases, and to the promoter of the *amtB-glnK-glnD* operon, encoding an ammonium transporter (28). These findings reveal cross talk between global regulators (PhoP, GlnR, and AfsR) in *S. coelicolor* that controls the expression of genes associated with secondary metabolite biosynthesis and is governed by a regulatory network of cross-talking global regulators (CTGRs). The CTGR network orchestrating cell nutritional and environmental stress responses modulates primary and secondary metabolism. Interestingly, no phosphate-related gene was found in the GlnR regulon, suggesting that GlnR has no direct effect on phosphate metabolism and demonstrating that the cross talk between GlnR and PhoP is not reciprocal (29).

Saccharopolyspora erythraea, a rare actinomycete species, has been used for industrial-scale production of erythromycin A, a broad-spectrum macrolide antibiotic against pathogenic Gram-positive bacteria. Nitrogen sources and phosphate concentration significantly influence erythromycin production (30); therefore, a detailed investigation to understand the nitrogen/phosphate utilization, metabolism, and regulation will be valuable to *S. erythraea* research. We previously identified 25 GlnR-dependent and GlnR-controlled transcription units (TUs) involving a total of 82 genes that are directly related to nitrogen utilization in *S. erythraea*. These genes are involved in ammonium uptake and assimilation, urea utilization, nitrite/nitrate assimilation, glutamate

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TABLE 1 Strains and plasmids used in this work

Strain or plasmid	Relevant characteristics or use	Source or reference
Strains		
S. erythraea NRRL23338	Parental strain, wild type	DSM 40517
E. coli DH5α	F^- ϕ80d*lacZ*ΔM (*lacZYA-argF*)*U169 deoR*	Invitrogen
E. coli BL21(DE3)-7101	The strain for expression of GlnR	Lab stock
E. coli BL21(DE3)	F′ *ompT* r_B^- m_B^- (λDE3)	Invitrogen
S. erythraea $phoP_O$	Overexpression of *phoP*; NRRL23338 integrated with pIB139-6965	This study
S. erythraea Δ*glnR*	NRRL23338 *glnR*::*tsr* (*glnR*-null mutant)	Lab stock
S. erythraea null mutant	Plasmid-alone negative control strain with pIB139 integrated into the genome	This study
S. erythraea Δ*glnR*/pIB-*glnR* ($glnR_C$)	*glnR*-complemented strain; Δ*glnR* strain carrying pIB-*glnR*	Lab stock
Plasmids		
pET28a(+)	Vector with T7 RNA polymerase-based promoter for expression in *E. coli* BL21; hexahistidine tag with thrombin cleavage	Thermo Scientific
p7101	pET28a(+) with *glnR* (SACE-7101) inserted in the NcoI-HindIII site	This study
p6965	pET28a(+) with *phoP* (SACE-6965) inserted in the NcoI-HindIII site	This study
$p6965^{130}$	pET28a(+) with $phoP^{DBD}$ (domain for DNA binding, 390 bp of C terminus) inserted in NcoI-HindIII	This study
pUC18-tsr	pUC18 with *tsr* gene for thiostrepton resistance inserted into the BamHI-SmaI site	32
pIB139	pSET152 with integrase of phiC31 and PermE, the strong promoter of *Streptomyces*	32
pIB139-7101	pIB139 with *glnR* inserted into NdeI-EcoRV	This study
pIB139-6965	pIB139 with *phoP* inserted into NdeI-EcoRV	This study

transport, arginine biosynthesis, nitric oxide biosynthesis, and transcriptional regulation and signal transduction associated with nitrogen source type and availability (31).

In this study, we identified the global regulator PhoP (SACE_6965), which controls seven TUs involved in phosphate metabolism in *S. erythraea*. The results revealed that PhoP and GlnR both collaboratively regulate the transcription of *glnR* and other nitrogen metabolism-related genes (*glnA1*, *glnA4*, *csbX*, *gltP*, *ureA*, and *gltB*). Furthermore, we found that GlnR negatively controlled phosphate metabolism through its binding to the promoter of *phoP-phoR* and that the two global regulators played reciprocal regulatory roles associated with nitrogen and phosphate metabolism in *S. erythraea.* Our findings present a new complex interconnected regulatory network involving GlnR/PhoP and significantly extend the understanding of regulation mechanisms associated with nitrogen/phosphate metabolism in actinomycetes.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. All strains and plasmids used in this work are listed in Table 1. Spores of *S. erythraea* strains were grown on agar plates in a medium containing 10 g/liter cornstarch, 10 g/liter corn steep liquor, 3 g/liter NaCl, 3 g/liter $(NH_4)_2SO_4$, 2 g/liter $CaCO_3$, and 20 g/liter agar in distilled H_2O (pH 7.2) at 30°C. *Escherichia coli* strains were grown at 37°C in liquid or on solid LB medium. All media were sterilized by autoclaving at 121°C for 30 min.

Construction of an *S. erythraea phoP*-overexpressing mutant. A *phoP* overexpression ($phoP_O$) mutant strain (YE6965) was generated by protoplast transformation with pIB139-6965 (Table 1) carrying a 681-bp fragment of the *phoP* open reading frame (SACE_6965) inserted between NdeI and EcoRV restriction sites (32). The plasmid pIB139-6965 was integrated into the *S. erythraea* genome using the phiC31 integrase. Apramycin resistance was examined following the addition of apramycin at a final concentration of 100 μg/ml, 24 h after transformation. The selected mutants were verified by PCR, real-time quantitative PCR (RT-qPCR), and DNA sequencing (PCR was used to amplify the fragment spanning the inserted *phoP* gene and its upstream and downstream plasmid regions using primers G6965F and G6965R; RT-qPCR was used to investigate the transcript levels of *phoP* with primers RT6965F and RT6965R). All primers used in this study are listed in Table 2. The negative control consisted of a plasmid-only construct that underwent protoplast transformation with pIB139 and was labeled "null." Our attempts to conduct experiments involving a *phoP*-null mutant strain were unsuccessful.

Overexpression and purification of the PhoP protein in *E. coli*. For heterologous expression of PhoP protein in *E. coli*, the *phoP* gene (SACE_6965) was amplified by PCR from the *S. erythraea* NRRL23338 genome using the primers PhoP_F (with the EcoRI restriction site) and PhoP_R (with the HindIII restriction site), and cloned into a pET-28a(+) vector in order to generate a His tag fusion protein. The pET-28a(+)-*phoP* plasmid (p6965) was transformed into *E. coli* BL21(DE3) cells and grown in LB medium at 37°C in an orbital shaker at 250 rpm to an optical density at 600 nm (OD_{600}) of 0.6. The expression of *phoP* was induced by the addition of isopropyl-β-D-1-thiogalactopyranoside at a final concentration of 0.1 mM and grown for an additional 6 to 8 h. Cells were harvested by centrifugation and washed twice with phosphate-buffered saline (PBS) (pH 8.0), and the cells were lysed by ultrasound. Cell debris and membrane fractions were separated from the soluble fraction by centrifugation at 12,000 × *g* for 45 min at 4°C. His-tagged PhoP (His_6-PhoP) was purified using nickel-nitrilotriacetic acid (Ni-NTA) Superflow columns (Qiagen, Valencia, CA, USA). The protein presented a maximal elution peak at ~250 mM imidazole in a buffer consisting of 50 mM NaH_2PO_4 and 300 mM NaCl (pH 8.0). Fractions containing His_6-PhoP were pooled and dialyzed in buffer D containing 50 mM Tris, 0.5 mM EDTA, 50 mM NaCl, 20% glycerol, and 1 mM dithiothreitol (DTT) (pH 8.0) at 4°C and stored at −80°C. Purity of the His_6-PhoP protein was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and protein concentration was determined using the Bradford reagent. The gene fragment encoding the C-terminal 130 amino acids of PhoP ($PhoP^{DBD}$) was amplified using the primers Pho^{DBD}_F and Pho^{DBD}_R. The heterologous expression and purification of $PhoP^{DBD}$ protein in *E. coli* were as described for the PhoP protein.

EMSA. The upstream regions (−300 to + 50) of *phoP*, *glnR*, and other PhoP-targeted genes were amplified by PCR using gene-specific primers containing the universal primer (5′-AGCCAGTGGCGATAAG-3′) sequence (Table 2) and biotin labeled by PCR using the 5′ biotin-modified

TABLE 2 Primers used in this work

Purpose and primer	Sequence (5′–3′)
PCR	
RT0175F	CCCCGTGCAGTGGGAGGT
RT0175R	GCGCGGAAGCGGTAGAAGTA
RT6716F	CTGGCTGCTGGAAGGTTTCC
RT6716R	TCAGGTCGTTGGCGTAGTGCT
RT7169F	GCCCGAGACCAAGCACCC
RT7169R	CGCCGCTGTTGCTGATGAG
RT6966F	CGCCAACGTCAGCCACG
RT6966R	AACGGCCCAGCGCCTC
RT7091F	CCAGATCAAGAAGTGGAACGACC
RT7091R	GAGCGGAAGAACGGGACGA
RT7099F	CAGATCAAGAAGTGGAACGACCC
RT7099R	GAGCGGAAGAACGGGACGA
RT6551F	GGCGAGAACGGCGAGC
RT6551R	GGGAACCTGATGCGGATGC
RT0658F	CCGACCTCGCCGACACCA
RT0658R	TACGCCCAGACCCCCTCC
RT7101F	GCAGGAGGTCTGGGGCTACG
RT7101R	GACGGGCGGACGAACTTGTAG
RT6965F	CGGTCAGGAGGCGTTGGAG
RT6965R	GCTGCTTGCAGACGTCGGT
RT0634F	ACGAGCGCGACAAGCTTCTC
RT0634R	ACCTGTTCGGCGGTCAGGAC
RT2830F	TGAAGATCCTCGGTGACCTGTT
RT2830R	CGACTCCATCTGCGTGATGC
RT5355F	GACAACCACGCGCTCTACAAGA
RT5355R	ATGAAGTGCTCCATCAGCGGGG
RT2507F	CGGCTACACCCCGCTCCCAC
RT2507R	GCTCCACACCACGCCCTTCG
RT6473F	AGGTGGTGGCGTATCCGAGCAT
RT6473R	GCGCGGAGTCGTCGAAAAGCTC
RT3798F	GTCTCCACTGTGGCCTCCTACA
RT3798R	CACAGTGGAGACGCCCATCT
RT8101F	GTTGCGATGCCGTGAGGT
RT8101R	CGGGTGTTACCGACTTTCA
G6965F	GAGGTACCGGTGATCATGG
G6965R	GTTGTGTGGAATTGTGAGCGG
PhoP_F	TAAGAATTCGTGACCAGGGTGCTGATCGTGG
PhoP_R	TAAAAGCTTTCACACCTCGAACTTGTAGCCGAG
Pho^{DBD}_F	CCGGAATTCGAGCTGATCGCCCGG
Pho^{DBD}_R	CCCAAGCTTTCACACCTCGAACTTGTAGCCG
EMSA	
PHO-boxF	GCCTTGTTCACCTGGGGTTCACCTCCAGGGCG
PHO-boxR	CGCCCTGGAGGTGAACCCCAGGTGAACAAGGC
Bio-primer	Biotin-AGCCAGTGGCGATAAG
7099F	AGCCAGTGGCGATAAGCGGCGATCAAGGTCTA
7099R	AGCCAGTGGCGATAAGGCGAGGAAGCCCAG
6551F	AGCCAGTGGCGATAAGTGCTGGGAGCTCGG
6551R	AGCCAGTGGCGATAAGACCACGACACCGAGG
6473F	AGCCAGTGGCGATAAGGTCGTTGTCGCCGAG
6473R	AGCCAGTGGCGATAAGGGCGTCGATGAGGAT
0175F	AGCCAGTGGCGATAAGCGGAGAGCGAACTGG
0175R	AGCCAGTGGCGATAAGGCCGAGCAGCACGC
6716F	AGCCAGTGGCGATAAGGTGTGACCTCCACCTTC
6716R	AGCCAGTGGCGATAAGGGTCCTGCGGGTCA
6965F	AGCCAGTGGCGATAAGCCAGGGCCGCTGAC
6965R	AGCCAGTGGCGATAAGCGCCCACGAGCAGC
7169F	AGCCAGTGGCGATAAGCGGCACCAATCACGA
7169R	AGCCAGTGGCGATAAGGCCAGCGCCGTCAG
0658F	AGCCAGTGGCGATAAGCATGCCGCGGGCG
0658R	AGCCAGTGGCGATAAGTTGAGGGAGGTGGCG
7169F	AGCCAGTGGCGATAAGCGGCACCAATCACGA
7169R	AGCCAGTGGCGATAAGGCCAGCGCCGTCAG
7101F	AGCCAGTGGCGATAAGCCGGTGTACAGCAAC
7101R	AGCCAGTGGCGATAAGTGCGGCAGCAGACCC
3998F	AGCCAGTGGCGATAAGCGGAGTCGCGTGTGG
3998R	AGCCAGTGGCGATAAGGGTCGTAGAGCCCCC
6473F	AGTGGCGATAAGGAGCCGGACCCCCAGC
6473R	AGTGGCGATAAGGCCGTGGCGTCGATGA
3095F	AGCCAGTGGCGATAAGGGTGAACTACGAGGT
3095R	AGCCAGTGGCGATAAGGTGGCGTCGAGATCG
2507F	AGTGGCGATAAGATTCCGCAAGCCCTCA
2507R	CGATAAGCAGCCGATCCGGTCGC
2830F	AGCCAGTGGCGATAAGCGCCGTCACTGTTTT
2830R	AGCCAGTGGCGATAAGGTGCCGACGACCATC
0634F	AGCCAGTGGCGATAAGATGATGCCTCGTCTC
0634R	AGCCAGTGGCGATAAGACGAGAAGCTTGTCG
5355F	AGCCAGTGGCGATAAGGCGGTGTCGTCGTTGT
5355R	AGCCAGTGGCGATAAGGCGCAGCTCCTCGAC

[a] Underlining indicates restriction enzyme sites.

universal primer. The PCR products were analyzed by agarose gel electrophoresis and purified using a PCR purification kit (Generay Biotech Co., Ltd., Shanghai, China). The concentration of biotin-labeled DNA probes was determined using a microplate reader (Biotek, Winooski, VT, USA). Electrophoretic mobility shift assays (EMSAs) were carried out according to manufacturer protocol for the chemiluminescent EMSA kit (Beyotime Biotechnology, Jiangsu, China). The binding reaction mixture contained 10 mM Tris-HCl (pH 8.0), 25 mM $MgCl_2$, 50 mM NaCl, 1 mM DTT, 1 mM EDTA, 0.01% Nonidet P40, 50 μg/ml poly[d(I-C)], and 10% glycerol. After binding, the samples were separated on a native PAGE gel in an ice bath containing 0.5× Tris-borate-EDTA at 100 V, and bands were detected using BeyoECL Plus (Beyotime Biotechnology). The amount of all DNA probes or double-stranded adapters used in the EMSA experiments was ~1 pmol, with the protein amount at ~10 pmol. The reactions were performed at 20°C for 15 min.

Computational analysis. The MEME/MAST tools (http://meme-suite.org/) and PREDetector (33) software were used to identify the GlnR/PhoP binding motif. Multiple alignment analysis of PhoP homologues was performed using ClustalX2 (34), and the phylogenetic tree was built using MEGA4 (35).

DNase I footprinting assay. DNase I footprinting assays were performed as previously described (36). The promoter region of *phoP* was PCR amplified using primers P6965F and P6965R (Table 2), and the amplicon was cloned into the T-vector pUC18B-T (Shanghai Biotechnology Corporation, Shanghai, China). The obtained plasmids were used as templates for further preparation of biotin-labeled probes using the universal primer (bio-Tprimer) (Table 2). After agarose gel electrophoresis, the 6-carboxyfluorescein (FAM)-labeled probes were purified using a QIAquick gel extraction kit (Qiagen) and quantified using a NanoDrop 2000C (Thermo Fisher, Waltham, MA, USA). For each assay, 200 ng of each probe was incubated with different amounts of His-PhoP or His-GlnR in a total volume of 40 μl in EMSA buffer (Beyotime Biotechnology). After incubation for 30 min at 25°C, 10 μl of solution containing ~0.015 units DNase I (Promega, Madison, WI, USA) and 100 nM freshly prepared $CaCl_2$ was added, and the sample was incubated for 1 min at 25°C. The reaction was stopped by adding 140 μl DNase I stop solution (200 mM unbuffered sodium acetate, 30 mM EDTA, and 0.15% SDS); the samples were extracted with phenol-chloroform and precipitated using ethanol, and the pellets were dissolved in 30 μl Milli-Q water. Preparation of the DNA ladder, electrophoresis, and data analysis were performed as

previously described (36), except that the GeneScan-LIZ500 size standard (Applied Biosystems, Foster City, CA, USA) was used.

Binding assays. All binding assays were performed in running buffer (10 mM Tris-HCl [pH 7.4], 150 mM NaCl, 2 mM TCEP [trichloroethyl phosphate], and 0.5 mg/ml bovine serum albumin [BSA]) using an Octet Red instrument (ForteBio, Inc., Menlo Park, CA, USA) at 25°C. Streptavidin-coated biosensors containing immobilized biotinylated double-stranded DNA (dsDNA) (PCR products of the upstream regions of *glnR* and *phoPR* obtained with Bio-primer, namely, P_{glnR} and P_{phoP}) were exposed to different concentrations of His_6-GlnR or His_6-PhoP. The equilibrium dissociation constants (K_Ds) associated with GlnR and PhoP were calculated and fitted to binding curves using the corresponding analysis software (ForteBio, Inc.).

RNA preparation and RT-PCR. Cell pellets were collected after 20 min of centrifugation at 4,000 × *g*. Total RNA was extracted using the RNAprep Pure cell/bacterium kit (Tiangen Biotech Co., Ltd., Beijing, China). RNA integrity was analyzed by 1% agarose gel electrophoresis, and RNA concentration was determined using a microplate reader (BioTek). Reverse transcription was undertaken using the PrimeScript RT reagent kit with gDNA Eraser (TaKaRa, Shiga, Japan). The RT-PCRs were performed in a 20-μl PCR solution from the SYBR premix Ex Taq GC kit (Perfect Real Time; TaKaRa, Japan) using ~100 ng cDNA as the template. All procedures were performed according to the manufacturer's instructions. PCR was conducted using a CFX96 real-time system (Bio-Rad, Hercules, CA, USA) with PCR conditions of 95°C for 5 min, then 40 cycles at 95°C for 5 s and 58 to 63°C for 30 s, and an extension at 72°C for 10 min.

Erythromycin determination. The method used for erythromycin determination by high-performance liquid chromatography was previously described (31).

RESULTS

SACE_6965 encodes the putative phosphate utilization regulator PhoP in *S. erythraea*. In order to investigate the phosphate control of metabolism, the protein sequences of the *S. coelicolor* two-component system, PhoP-PhoR (SCO4230-SCO4229), were used as query sequences to aid in the identification of their orthologues in *S. erythraea*. SACE_6965 and SACE_6966 were identified as the closest homologues of SCO4230-SCO4229 (see Fig. S1 in the supplemental material). The results revealed a high similarity throughout the full-length sequence (especially in DNA recognition helical regions) and, therefore, suggested that SACE_6965 encodes the putative phosphate utilization regulator PhoP, which plays an important role in regulating gene expression in response to phosphate availability in *S. erythraea*.

The upstream regions of the genes involved in phosphate metabolism have been reported to contain a putative PhoP-binding motif (PHO box) in different *Streptomyces* strains, with a typical PHO box consensus being identified. The PHO box is formed by two conserved direct repeat units (DRUs) of 11 nucleotides in actinomycetes, with each one formed by an identical five-nucleotide sequence (GT/GTCA) followed by a six-nucleotide less-conserved tail region, and bound by a protein monomer (12, 22). Based on the *Streptomyces* PHO box motif, we searched putative PhoP-binding sites in upstream of all the *S. erythraea* orthologues of known genes involved in the uptake, assimilation, and metabolism of phosphate in actinomycetes. Combined utilization of MAST/MEME tools and the PREDetector software identified putative PHO box sequences in the upstream regions of eight transcription units (TUs) associated with phosphate metabolism (Fig. 1a). These include genes involved in phosphate transport (the *phoS2-pstCAB* operon; the SACE_6551-SACE_6552 operon, encoding phosphate-binding proteins; and *phoU*, encoding the phosphate-specific transport system accessory protein PhoU), glycerophospholipid metabolism (*glpD*, encoding glycerol-3-phosphate dehydrogenase, and *glpQ*, encoding glycerophosphoryl diester phosphodiesterase), phosphate uptake (*phoD1* and *phoD2*, coding for alkaline phosphatase D), and the two-component system (SACE_6965-SACE_6966). EMSA experiments with purified SACE_6965 were performed, revealing their binding to the upstream promoter regions of eight TUs (Fig. 1b). In order to confirm the PHO box sequences, the 5′ biotin-labeled synthetic dsDNA fragments containing portions of the predicted PhoP-binding motif and mutant motif (in the upstream region of the *phoS2-pstCAB* operon) were used for the EMSA. The results showed that the native PhoP-binding motif was shifted by purified *S. erythraea* SACE_6965. However, the shifted bands of the mutant fragments were evidently weakened (Fig. 1c). Taken together, these results indicate that SACE_6965 is the closest homologue of the phosphate regulator PhoP studied in *Streptomyces* spp., and we propose naming it PhoP.

The transcription levels of genes containing a putative PHO box in response to phosphate availability were investigated using phosphate downshifted cultures. The *S. erythraea* wild-type strain NRRL23338 was grown in high-phosphate MG medium (MG-10 mM, 60 g/liter starch, 60 mM glutamate, and 10 mM phosphate) for 40 h and then transferred to low-phosphate medium (MG-50 μM) for 12 h. Two culture samples were collected before and after the downshift in order to isolate mRNA for RT-PCR. As expected, the *phoP-phoR* operon was strongly induced, while expression of most target genes was also upregulated under low-phosphate conditions. The quantitative RT-PCR experiments indicated that transcript levels significantly increased after the downshift: 410-fold for *phoD2*, 30-fold for *phoU*, 25.5-fold for *phoS2*, 20-fold for *phoP*, 13.4-fold for *phoR*, and 13.5-fold for *glpQ* (Fig. 1d). The most strongly induced gene was *phoD1* (410-fold increase), which encodes alkaline phosphatase D and is responsible for removing phosphate groups from many types of phosphate-containing molecules prior to uptake. No change in *glpD* and *ptsS* transcript levels was observed. To further examine the regulatory role of PhoP on the expression of identified target genes, we constructed a *phoP*-overexpressing ($phoP_O$) mutant strain exhibiting an ~4-fold increase in gene expression (see Fig. S2 in the supplemental material). The overexpression of *phoP* resulted in a 2- to 11-fold (11.5-fold for *phoD1*, 4-fold for *phoS2*, 4.3-fold for *glpQ*, 2.5-fold for *phoD*, and 2.2-fold for *phoD2*) increase in induction of target genes (Fig. 1e). A similar result was observed in the wild-type strain responding to phosphate starvation. These results further demonstrate that PhoP exhibits a regulatory function as a transcriptional activator of these target genes involved in phosphate metabolism.

PhoP directly controls the transcription of *glnR* and other GlnR-regulated genes involved in nitrogen metabolism. Previously, we reported that the OmpR-type response regulator GlnR (encoded by SACE_7101) was a central global regulator of nitrogen metabolism in *S. erythraea* (31). A typical PHO box (two DRUs, GTTCGCCTTCT and GTTCACAATTGT) was observed in the upstream region (from position −182 to −161, relative to the translation start site in the antisense strand) of the *glnR* gene and separated by only 4 bp from the GlnR-binding motif (Fig. 2a). In order to determine whether the PhoP protein binds directly to the promoter regions of the *glnR* gene, gradient EMSAs were performed using purified His-PhoP protein. To assess binding spec-

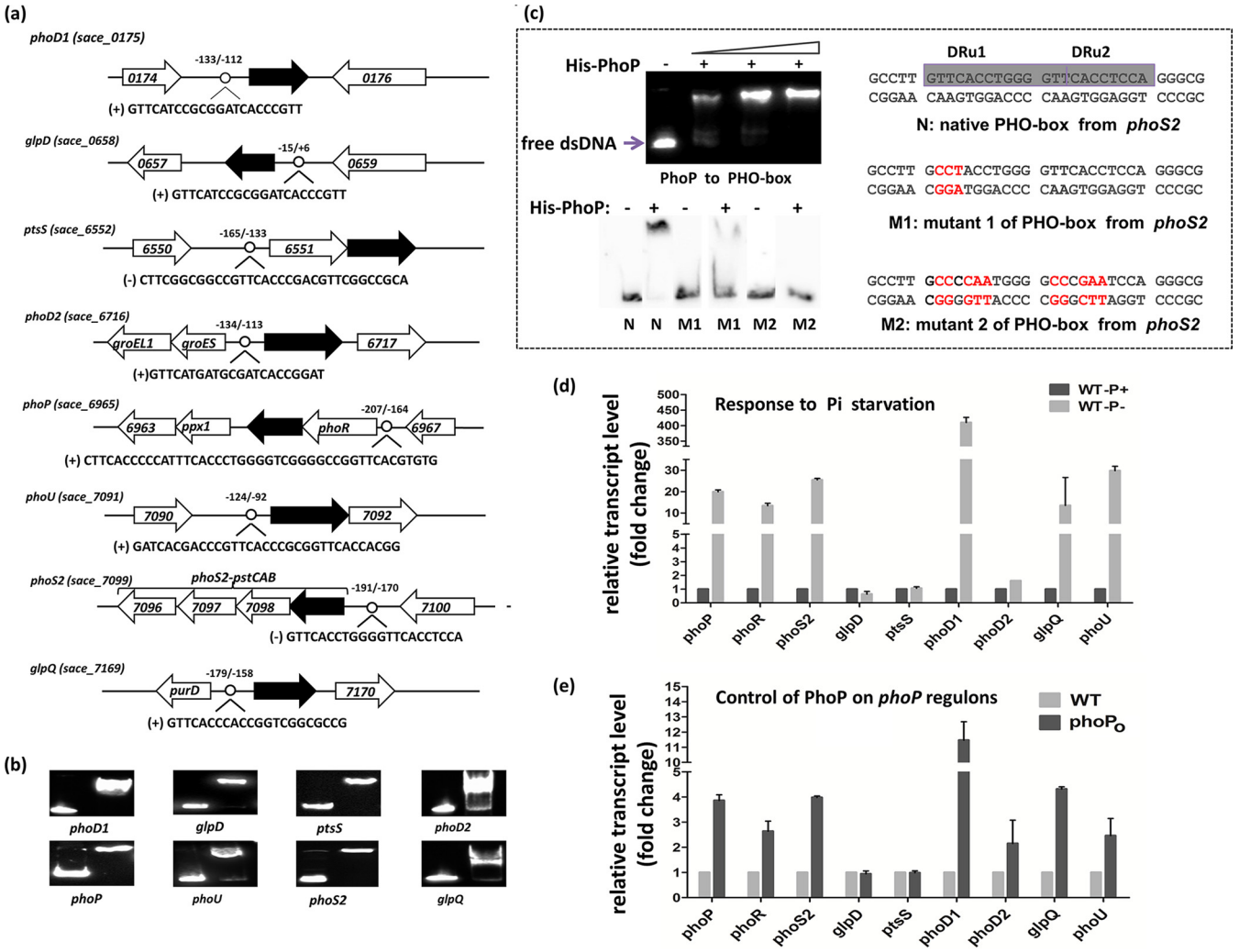

FIG 1 PhoP regulates genes involved in phosphate metabolism. (a) PhoP-binding motifs were identified in phosphate-related genes. (b) EMSA results indicating His-PhoP interactions with the predicted motifs. The DNA probes containing the predicted motifs were incubated with 1 μM protein and a 200-fold excess of nonspecific competitor DNA (sperm DNA). (c) EMSA results showing interactions between His-PhoP and the PHO box or mutant PHO box in the *phoS2* gene. (d) Transcription profiles of *phoPR* and PhoP-regulated genes in response to phosphate downshift. (e) Transcription profiles of *phoPR* and PhoP-regulated genes in response to overexpression of *phoP* gene.

ificity, an unlabeled specific probe (100-fold) or nonspecific competitor DNA (100-fold; salmon sperm DNA) was used. Obvious shifted bands were observed in all EMSAs following addition of His-PhoP (Fig. 2b). The results indicated that the DNA fragment from the *glnR* upstream region containing the predicted PhoP binding site was bound by His-PhoP, thus suggesting that global nitrogen regulator GlnR in *S. erythraea* is subject to transcriptional regulation by the phosphate regulator PhoP.

To elucidate the regulatory effects of PhoP on the *glnR* gene in *S. erythraea*, *glnR* transcription levels were compared with those of the wild-type and $phoP_O$ mutant strains in nitrogen-rich tryptic soy broth (TSB) medium and nitrogen-limited conditions (minimal Evans medium supplemented with 2 mM glutamine). As shown in Fig. 2c, *phoP* overexpression resulted in a 4.4-fold increase in *glnR* induction in the nitrogen-rich medium, whereas only a 1.6-fold increase in *glnR* induction was seen in the nitrogen-limited medium. No changes in erythromycin production or *glnR* or *phoP* transcript levels were observed in the plasmid-only negative control (see Fig. S3 in the supplemental material). These results confirm that PhoP enhances *glnR* expression in *S. erythraea*.

PhoP exerts direct and indirect regulatory effects on nitrogen metabolism in *S. coelicolor* (25). By searching for promoter regions containing a consensus PhoP-binding motif, we found that many genes involved in nitrogen metabolism are also regulated by PhoP in *S. erythraea*. The genes or operons having PHO boxes in their promoter regions include *glnA3* (SACE_3095; two DRUs [GTTCACCCGCT and GGTCCTCCTCG]) and *glnA4* (SACE_5355; two DRUs [GGTCACCAGAT and GTTCCTCCCGG]), which encode two glutamine synthetases; *ureABCFGD* (SACE_0634 to -0639; two DRUs [TTGCGGACTTT and TTTCCAACATT]), which encode enzymes involved in urea utilization; *gltP* (SACE_2830; two DRUs [GTTCACACGGC and GGTCACGTTGG]), which encodes a Na^+/glutamate:H^+ symporter; *gltB-gltD* (SACE_3998 and SACE_3997; two DRUs [GGTCAGCGGGG and TCGC CGCCGTT]), which encode glutamate synthase (NADPH); and *csbX* (SACE_2507; two DRUs [CCTCAACCGTG and GGTCACC

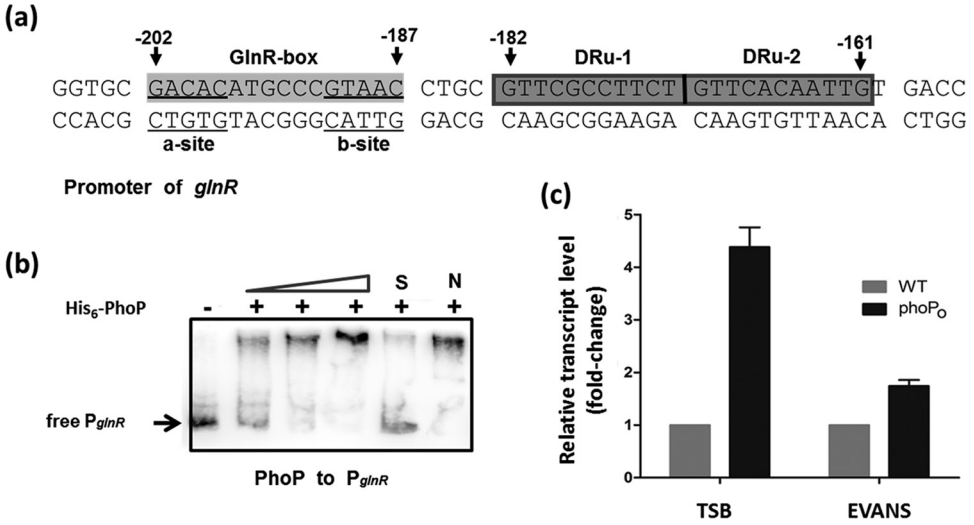

FIG 2 PhoP controls *glnR* transcription. (a) Putative PhoP-binding sites in the *glnR* promoter region. (b) EMSA results indicating His-PhoP interactions with the *glnR* upstream promoter region; unlabeled specific probe (100-fold) (S) or nonspecific competitor DNA (100-fold, salmon sperm DNA) (N) was added. (c) The positive regulation of PhoP on the transcription of *glnR* in TSB and EVANS-15 mM glutamine. Changes represent the level of expression in the *phoP* overexpression mutant ($phoP_O$) relative to the expression levels observed in the wild-type strain. Results were normalized to 16S rRNA levels. Data are means and standard deviations from three independent experiments, each with triplicate samples, using distinct cDNA preparations for each RNA sample.

GGCC]), which encodes α-ketoglutarate permease (Fig. 3a). All of these genes were identified as being targeted by GlnR in our previous study (31). Similar to *S. coelicolor* PhoP, *S. erythraea* PhoP can exert control of the nitrogen metabolism through its binding to the promoter of the *glnR* gene. Moreover, PhoP exerts similar effects on ammonium assimilation, via glutamine synthetase/glutamate synthase (GS/GOGAT), through direct binding to the promoters of *glnA*, *glnA4*, and *gltB-gltD*, whereas PhoP appears to have no effect on the uptake of ammonium (*amt* operon). Additionally, *S. erythraea* PhoP exerts a regulatory effect on the uptake of glutamate (*gltP*) and its precursor α-ketoglutarate (*csbX*), as well as urea utilization (*ureABCFGD*).

To determine whether PhoP binds to the promoter regions of these genes, EMSAs were performed using purified His-PhoP with biotin-labeled DNA probes (representing the upstream regulatory regions of each gene from −250 to +50) containing all of the predicted PhoP motifs. The results confirmed that PhoP binds to these regions (Fig. 3b). RT-PCR experiments indicated that the overexpression of PhoP resulted in the induction of *gltP* (21.4-fold), *gltB* (8.5-fold), *ureA* (1.8-fold), and *glnA1* (1.5-fold) in TSB medium. Meanwhile, the transcription levels of *glnA4* and *csbX* significantly decreased in the $phoP_O$ mutant by factors of 27-fold and 13-fold, respectively (Fig. 3c). In contrast, *S. coelicolor* PhoP revealed direct and indirect negative control of *glnR*, *glnA*, *glnII*, and *amtB* expression, whereas no positive induction of nitrogen-related genes was observed (24, 28).

GlnR reciprocally controls the transcription of the *phoP* gene in *S. erythraea*. There is nonreciprocal regulation of the GlnR-regulated genes by the PhoP protein in *S. coelicolor* and *S. lividans* (25). PhoP directly controls at least seven nitrogen metabolism-related genes, while GlnR does not regulate the phosphate regulon genes (29). However, we found that *S. erythraea* GlnR binds to the *phoPR* operon promoter (Fig. 4a). Shifted bands were observed for all EMSAs when the His-GlnR protein was added. In order to identify the exact DNA sequences that bound GlnR in the *phoP* promoter region, a DNase I footprinting assay using purified recombinant His-GlnR and a fluorescent FAM-labeled probe was performed. With the addition of His-GlnR (3 μg), a protected region of 17 nucleotides was detected from position −75 to −91, relative to the translation start site in the *phoPR* promoter coding strand (Fig. 4b). These results revealed that the GlnR-binding site is located in close proximity to the PHO box (separated by only 2 bp), indicating that the two regulators may competitively bind to the adjacent binding sites (Fig. 4c).

In order to determine the affinities of GlnR and PhoP for the upstream regions of the *phoP* and *glnR* genes, an interferometry assay was performed using the Octet system (ForteBio, Inc.). The K_D was determined by immobilizing biotin-modified DNA fragments with increasing amounts of purified regulator proteins. We found that GlnR and PhoP exhibited different affinities (about 2- to 5-fold) for P_{phoP} and P_{glnR}. Purified His-GlnR and His-PhoP bound to P_{glnR} with K_Ds of ~203 nM and ~1.06 μM, respectively, and bound to P_{phoP} with K_Ds of ~749 nM and ~198 nM, respectively. GlnR had a 5-fold-higher affinity for P_{glnR} than PhoP, while PhoP had a 3.8-fold-higher affinity for P_{phoP} than GlnR.

In order to investigate whether GlnR and PhoP interfere with the binding of one another to P_{glnR} and P_{phoP}, competitive EMSAs were conducted using His-GlnR and His-PhoP both together and separately. It was difficult to discriminate between the shifted bands of GlnR and PhoP in EMSA experiments, given their similar molecular weights; however, we found that His-$PhoP^{DBD}$ (protein containing only the DNA binding domain, amino acids 97 to 226) and His-PhoP displayed similar binding affinities for the PHO box (see Fig. S4 in the supplemental material). As shown in Fig. 5a, His-GlnR binds to P_{glnR} to produce shifted bands (Fig. 5a, lanes 2 to 4) in the absence of His-PhoP. In the absence of His-GlnR, the shifted bands representing P_{glnR}-$PhoP^{DBD}$ (Fig. 5a, lanes 5 to 7) were observed with increasing concentrations of His-$PhoP^{DBD}$. However, the addition of two transcription factors to the *in vitro* reaction resulted in loss or attenuation of the shifted bands corre-

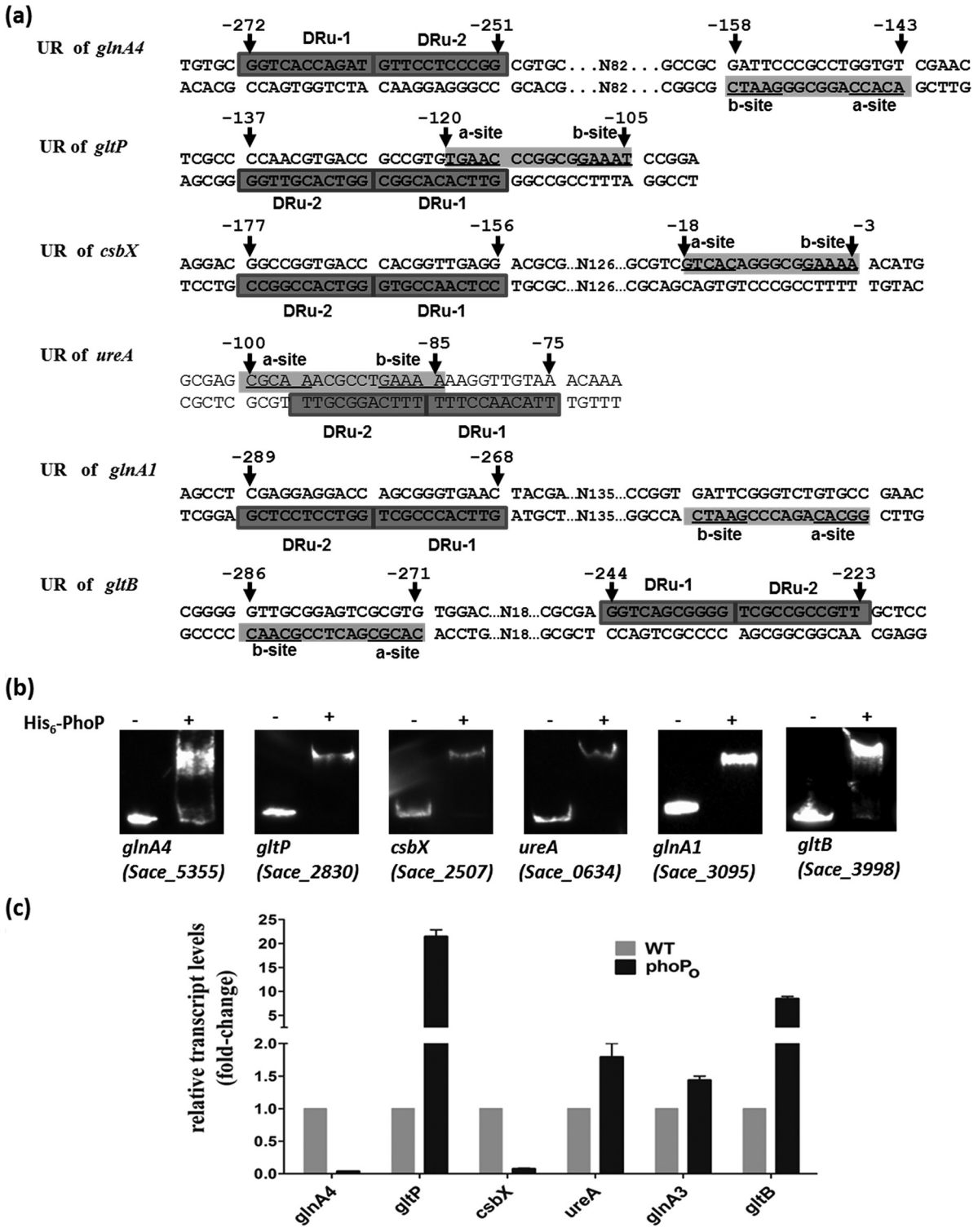

FIG 3 PhoP regulates genes involved in nitrogen metabolism in *S. erythraea*. (a) Predicted GlnR and PhoP binding motifs in the upstream regions (URs) of nitrogen-related genes. (b) EMSA results indicating interactions between His-PhoP and the upstream regions of PHO box-containing genes following incubation with biotin-labeled DNA with 1 μM protein and a 200-fold excess of nonspecific competitor DNA. (c) RT-PCR data. Changes represent expression levels in the wild-type strain relative to the expression levels observed in the $phoP_O$ mutant.

sponding to P_{glnR}-GlnR and P_{glnR}-$PhoP^{DBD}$, resulting in an additional band shifted to a greater degree, corresponding to P_{glnR}-GlnR/$PhoP^{DBD}$ complex (a complex of both factors) (Fig. 5a, lanes 8 to 10). Similarly, in the presence of both His-GlnR and His-PhoP, the shifted bands corresponding to P_{phoP}-GlnR and P_{phoP}-$PhoP^{DBD}$ disappeared, whereas the largest shifted band representing P_{phoP}-GlnR/$PhoP^{DBD}$ complex was observed (Fig. 5b). These results demonstrated that both transcription factors were

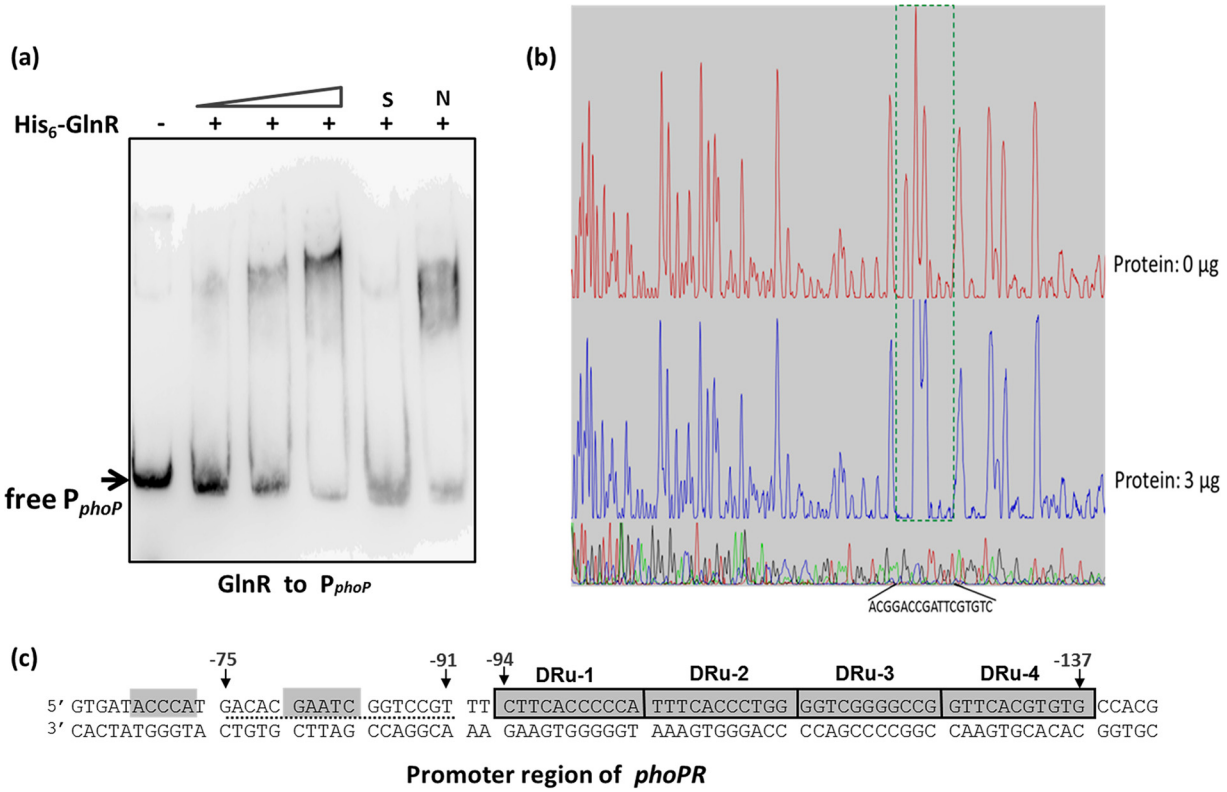

FIG 4 GlnR binds to the *phoPR* promoter and controls its transcription. (a) EMSA using 1 μM purified *S. erythraea* His_6-GlnR protein and 1 pmol biotin-labeled PCR products from the upstream region of operon *phoPR*. A 200-fold excess of nonspecific competitor DNA was included in every lane as an internal control to avoid unspecific binding of the protein to the DNA. (b) DNase I footprinting assays for the GlnR-P_{phoPR} complexes. (c) The protected DNA sequences located in upstream region of *phoPR*. The black dotted line indicates the respective footprints of GlnR.

able to simultaneously bind to the promoter regions of their respective genes.

To further elucidate the regulatory effects of GlnR on *phoP* transcription in *S. erythraea*, we constructed a *glnR* deletion mutant (Δ*glnR*) and a *glnR*-complemented ($glnR_C$) strain using methods described previously (31, 37). Three strains were cultivated in TSB medium, cells were harvested for RNA extraction at three time points (24 h, 36 h, and 48 h), and *phoP* transcription was analyzed using RT-PCR. As shown in Fig. 6a, ~2-fold increases in *phoP* transcription were observed in the Δ*glnR* mutant strain, indicating that *phoP* was repressed by GlnR. Complementation of the *glnR* gene restored this repressive effect. We also examined the effect of GlnR on *phoP* transcription in response to nitrogen availability. *S. erythraea* wild-type strains were grown for 2 days at 30°C in TSB medium and then collected and washed twice with normal saline. The cell pellets were added to Evans medium with glutamine as the nitrogen source. RT-PCR experiments revealed that *phoP* transcription levels were enhanced 6-fold in the high-nitrogen medium relative to the levels observed in the low-nitrogen medium (Fig. 6b). It was also observed that the nitrogen-mediated effect on *phoP* expression disappeared in the Δ*glnR* strain and was recovered in the $glnR_C$ strain (Fig. 6c). These results demonstrate that GlnR represses *phoP* transcription in response to nitrogen availability in *S. erythraea*. Next, we examined the transcript levels of other genes (*glnR*, *ureA*, *phoP*, and *phoS2*) involved in nitrogen and phosphate metabolism in nitrogen- and phosphate-limited or -rich media. As expected, nitrogen-related genes (*glnR* and *ureA*) were induced about 2.5- to 10-fold under nitrogen-limited conditions (Fig. 6b), while phosphate-related genes (*phoP* and *phoS2*) were induced about 22- to 27-fold under phosphate-limited conditions (Fig. 6d). Low phosphate also resulted in the induction of the genes *glnR* and *ureA* (about 2- to 4-fold), as the target genes of PhoP. However, we found that the phosphate-related gene *phoS2*, which is not a direct target gene of GlnR, was repressed under nitrogen-limited conditions (Fig. 6b). Taken together, the results indicate that there appears to be reciprocal regulatory cross talk between GlnR and PhoP in *S. erythraea*, unlike *S. coelicolor* and *S. lividans*, indicating multiple complex regulatory processes associated with response to external nutrition conditions, such as nitrogen and phosphate concentrations. This PhoP-GlnR regulatory circuit, which includes a positive and a negative interaction, leads to homeostasis (Fig. 6e).

Impact of nitrogen and phosphate status on antibiotic biosynthesis. Finally, we investigated the effect of GlnR and PhoP on *S. erythraea* growth and erythromycin production. The growth curves observed for the wild type (WT), the *glnR* deletion (Δ*glnR*) mutant, the *glnR*-complemented ($glnR_C$) strain, the *phoP*-overexpressing mutant ($phoP_O$), and the *phoP*-overexpressing Δ*glnR* (Δ*glnR* $phoP_O$) strain in TSB medium are presented in Fig. S5A in the supplemental material. No change of growth in all five strains was observed. Notably, both regulators exerted an impact on erythromycin production; specifically, production of erythromycin in the Δ*glnR* and $phoP_O$ mutants increased by ~23% and

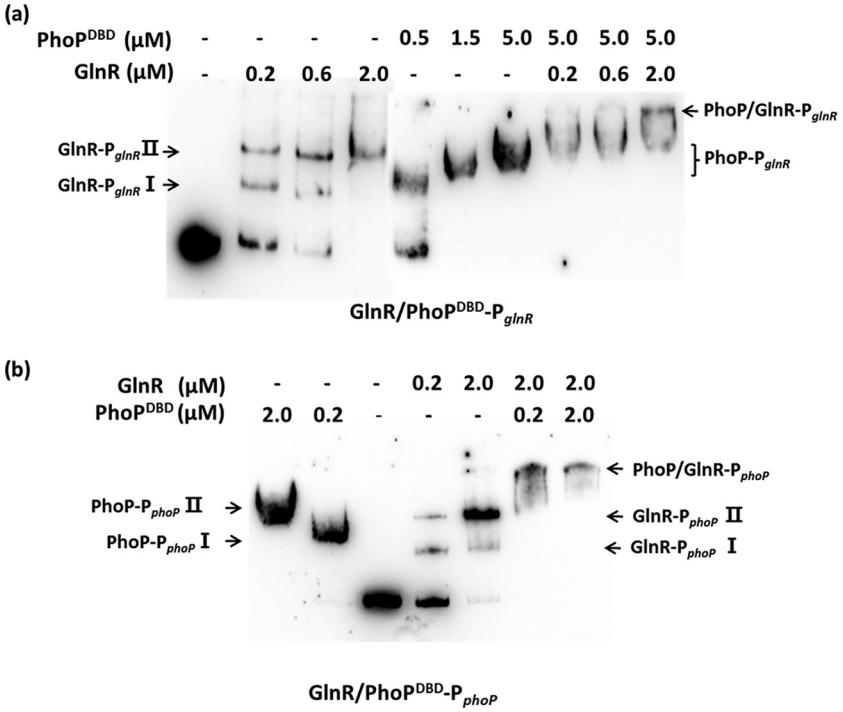

FIG 5 Interaction of both PhoP and GlnR with promoter regions. (a) Competitive EMSA results indicating the binding of His_6-GlnR and His_6-PhoP^{DBD} to the *glnR* promoter (P_{glnR}). (b) Competitive EMSA results indicating the binding of His_6-GlnR and His_6-PhoP^{DBD} to the *phoP* promoter (P_{phoP}).

~40%, respectively, compared to the wild-type strain, with further improvement by ~56% observed in the Δ*glnR phoP*$_O$ strain (see Fig. S5B in the supplemental material). The results revealed that GlnR repressed whereas PhoP activated erythromycin production, thereby demonstrating the importance of nitrogen/phosphate source sensing/utilization in *S. erythraea* antibiotic production.

DISCUSSION

Here, we identified PhoP as a global regulator of phosphate utilization in *S. erythraea* and showed the reciprocal cross talk between PhoP and the nitrogen regulator GlnR, suggesting a reciprocal regulatory GlnR/PhoP network in coordinating metabolic response according to nitrogen/phosphate availability. PhoP negatively controls ammonium uptake and assimilation by repressing the transcription of *glnR*, *glnA*, *glnII*, and the *amtB-glnK-glnD* operon in *S. coelicolor* (24). The regulatory effects of *S. erythraea* PhoP on nitrogen metabolism revealed three distinct features: (i) *S. erythraea* PhoP can exert positive control of nitrogen metabolism through its binding to the promoter of *glnR* gene; (ii) PhoP exerts enhanced ammonium assimilation of glutamine synthetase/glutamate synthase (GS/GOGAT) through direct binding to the promoters of *glnA*, *glnA4*, and *gltB-gltD*, while PhoP has no effect on the uptake of ammonium (*amt* operon); (iii) *S. erythraea* PhoP exerts regulatory effects on the uptake of glutamate (*gltP*) and its precursor α-ketoglutarate (*csbX*), as well as utilization of urea (*ureABCFGD*). Interestingly, GlnR- and PhoP-binding sites were identified in the promoter regions of both *glnR* and *phoP* in *S. erythraea*. Unlike the nonreciprocal regulation of GlnR and PhoP in *S. coelicolor*, *S. erythraea phoP* and *phoR* genes were found in the GlnR regulon, suggesting that GlnR exerts a direct regulatory effect on phosphate metabolism and demonstrating that the cross talk between GlnR and PhoP is reciprocal. It is worth noting that two binding sites in the *glnR* and *phoP* promoter regions are located in close proximity to one another (separated by only 2 to 4 bp), suggesting that the two proteins may competitively bind to their adjacent binding sites. However, the experimental results revealed that the binding of GlnR and PhoP to their respective sites occurred independently of one another without any interference. Both regulators collaboratively controlled nitrogen and phosphate metabolism in response to environmental changes. In summary, these observations demonstrated that *S. erythraea* has an extensive cross talk mechanism between regulatory systems involved in the global coordination of nitrogen and phosphate metabolism (Fig. 7).

Phosphorus and nitrogen are essential components of microbial nutrition. Soil microorganisms frequently encounter limited supplies of phosphate and nitrogen. The regulation of GlnR-related metabolism in soil microorganisms is under PhoP control, indicating that phosphate starvation triggers the PhoP/GlnR-mediated response, resulting in the expression of genes involved in nitrogen and phosphate metabolism. However, GlnR directly represses *phoP* transcription, indicating that nitrogen starvation results in the repression of genes associated with phosphate metabolism. The reciprocal cross-regulation of phosphate and nitrogen metabolism may be explained as a mechanism for saving cell resources in order to ensure that *S. erythraea* obtains nitrogen (may nitrogen control be implemented first in *S. erythraea*?). However, PhoP negatively regulates *glnR* and other nitrogen-related genes (*glnA*, *glnII*, and *amtB-glnK-glnD*) in *S. coelicolor*, suggesting that phosphate limitation reduces transcription of genes involved in nitrogen utilization (may phosphate control be implemented first

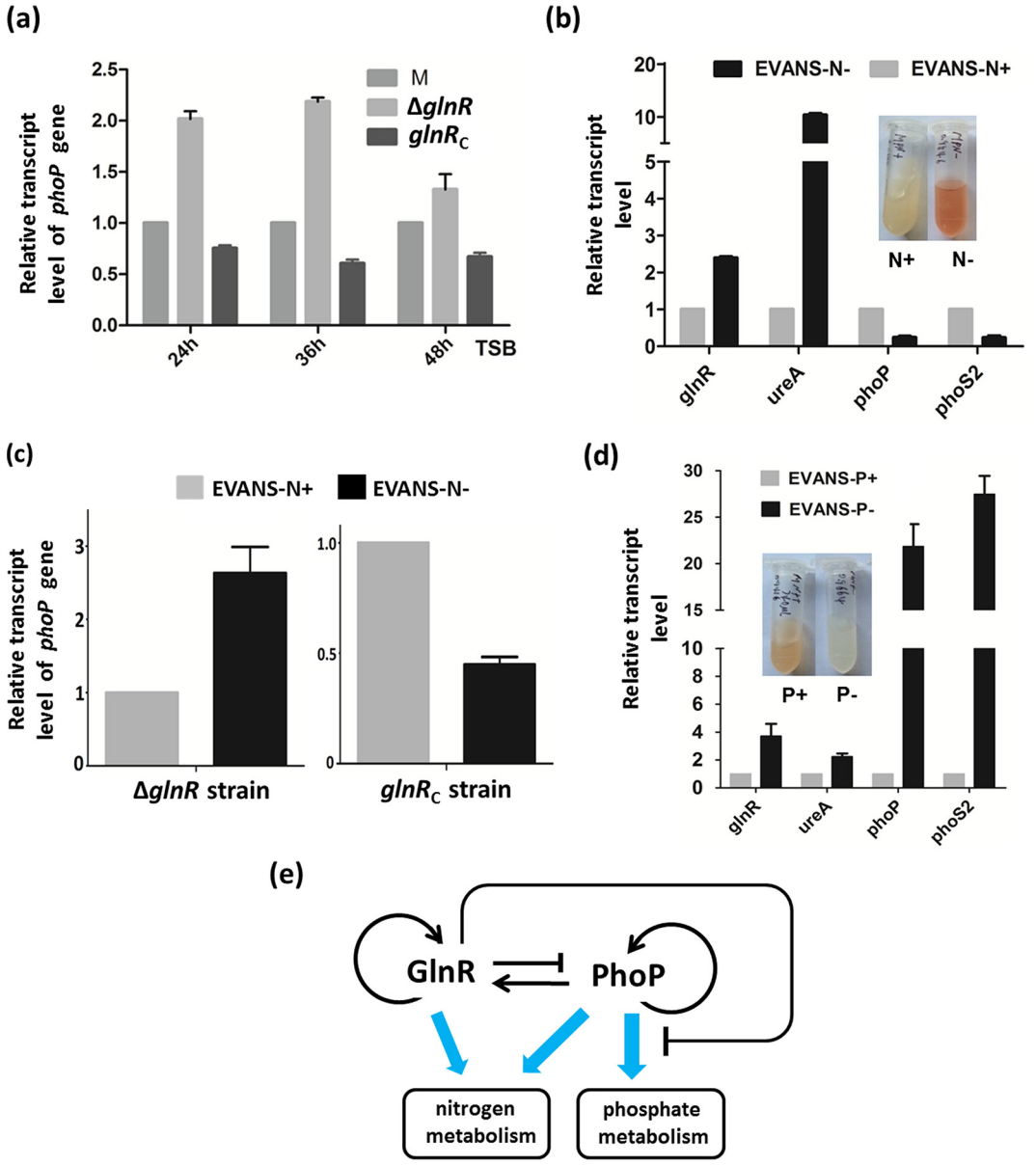

FIG 6 Transcription levels of genes involved in nitrogen/phosphate metabolism in response to nutrient availability. (a) Differences in *phoP* transcription between the wild-type, Δ*glnR*, and $glnR_C$ strains in TSB medium. RNAs were extracted from *S. erythraea* NRRL2338 and the Δ*glnR* and $glnR_C$ strains grown in TSB at 24 h, 36 h, and 48 h. Results were normalized to 16S rRNA levels. (b) Transcription profiles of genes (*glnR*, *ureA*, *phoP*, and *phoS2*) under nitrogen-rich conditions (i.e., in Evans medium with 30 mM glutamine [Evans-N+]) or nitrogen-limited conditions (i.e., in Evans with 2 mM glutamine [Evans-N−]). (c) Transcription profiles of *phoP* in Δ*glnR* and $glnR_C$ strains in Evans-N+ or Evans-N−. (d) Transcription profiles of genes (*glnR*, *ureA*, *phoP*, and *phoS2*) under phosphate-rich conditions (i.e., in Evans medium with 10 mM K_2HPO_4 [Evans-P+]) or phosphate-limited conditions (i.e., in Evans medium with 40 μM K_2HPO_4 [Evans-P−]). Fold changes represent gene expression levels in Evans-N+ or -P+ relative to the expression levels observed in Evans-N− or -P−. Data are means and standard deviations from three independent experiments, each with triplicate samples, using distinct cDNA preparations for each RNA sample. (e) The PhoP-GlnR regulatory circuit, which includes a positive and a negative interaction.

in *S. coelicolor*?). The differential control of PhoP (positive and negative) on nitrogen metabolism in *S. erythraea* and *S. coelicolor* indicates that actinomycetes have evolved several nutritional regulation networks to adapt to changes in the availability of nitrogen and phosphate nutrients in their various ecological niches.

Knowledge about nitrogen and phosphate metabolism and control in the erythromycin producer *S. erythraea* is sparse. The choice of nitrogen/phosphate source and its concentration have a great influence on the erythromycin production (30). Reeve and Baumberg investigated the effect of glucose, nitrogen, and phosphorus sources on the timing and extent of erythromycin production (30). The erythromycin production and erythromycin biosynthesis gene (*ery*) mRNAs were detectable in low-phosphate (<1 mM) cultures. High levels of phosphate (10 to 100 mM) in cultures repressed erythromycin synthesis and the transcription of *ery* genes. These results suggest that ammonium and phosphate impact the transcription of *ery* cluster genes and that nitrogen/phosphate metabolism and synthesis of erythromycin are deeply

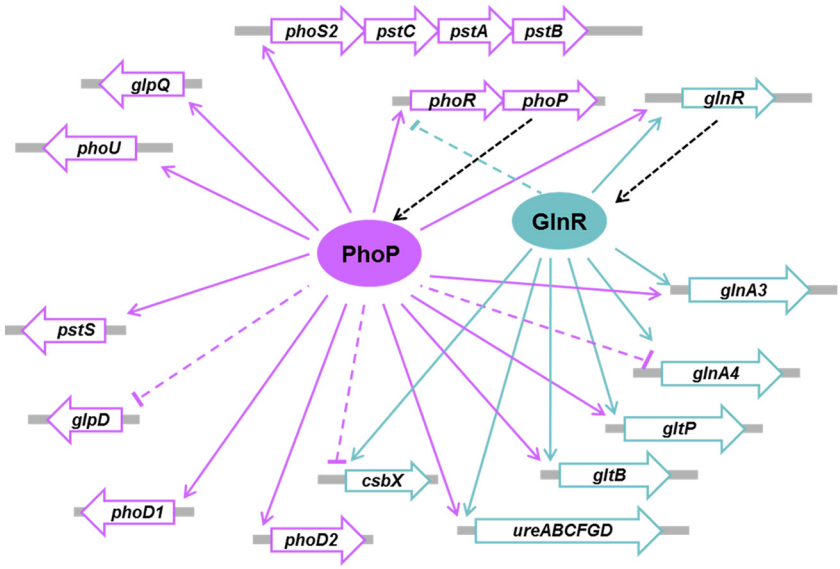

FIG 7 Networks of GlnR and PhoP in *S. erythraea*.

interconnected. This conclusion was also supported by results of recent experiments showing that erythromycin production is strongly inhibited by ammonium (38). These observations provide evidence that *S. erythraea* may possess a molecular mechanism involving cross talk between nitrogen/phosphate metabolism and erythromycin synthesis.

In our study, the deletion of *glnR* increased erythromycin production; however, when the *glnR* gene was reintroduced into the Δ*glnR* mutant, production of erythromycin decreased to levels comparable to that of the wild-type strain (see Fig. S5B in the supplemental material). The deletion of the *glnR* gene in *S. coelicolor* and *Amycolatopsis mediterranei* results in reduced antibiotic production (19, 39). The PhoR-PhoP system is involved in regulating the production of actinorhodin in *S. lividans* (12), undecylprodigiosin in *S. coelicolor* (40), and pimaricin in *Streptomyces natalensis* (41). Martin et al. found that PhoP regulatory effect on antibiotic biosynthesis may be exerted through signaling cascades of PhoP-AfsS-AfsR-SARP (ActII-orf4 or RedD) in *Streptomyces* (25). However, the gene homologous to *afsS* was not found in the *S. erythraea* genome, and no SARP was identified as being responsible for erythromycin biosynthesis. Interestingly, erythromycin production in the $phoP_O$ strain increased by 40% relative to the wild-type strain, suggesting that PhoP also regulates erythromycin biosynthesis in *S. erythraea*. Such observations suggest that GlnR and PhoP both may play a role in the regulation of *S. erythraea* antibiotic production; however, it remains unclear how GlnR/PhoP-mediated regulation is connected to antibiotic production. These nutrient-sensing regulators may be important for the induction of a general stress response, triggered by nutrient limitation, which finally activates antibiotic biosynthesis. Deeper knowledge of nitrogen/phosphate metabolism could provide a better understanding of the link between primary and secondary metabolism in erythromycin-producing microorganisms.

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