



# Physiological Roles of the Dual Phosphate Transporter Systems in Low and High Phosphate Conditions and in Capsule Maintenance of *Streptococcus pneumoniae* D39

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Unlike most bacteria, *Streptococcus pneumoniae* (pneumococcus) has two evolutionarily distinct ABC transporters (Pst1 and Pst2) for inorganic phosphate ( $P_i$ ) uptake. The genes encoding a two-component regulator (PnpRS) are located immediately upstream of the *pst1* operon. Both the *pst1* and *pst2* operons encode putative PhoU-family regulators (PhoU1 and PhoU2) at their ends. This study addresses why *S. pneumoniae* contains dual  $P_i$  uptake systems and the regulation and contribution of the Pst1 and Pst2 systems in conditions of high (mM)  $P_i$  amount and low ( $\mu$ M)  $P_i$  amount. We show that in unencapsulated mutants, both *pst1* and *pst2* can be deleted, and  $P_i$  is taken up by a third  $Na^+/P_i$  co-transporter, designated as NptA. In contrast, either *pst1* or *pst2* is unexpectedly required for the growth of capsule producing strains. We used a combination of mutational analysis, transcript level determinations by qRT-PCR and RNA-Seq, assays for cellular PnpR~P amounts by SDS-PAGE, and pulse- $P_i$  uptake experiments to study the regulation of  $P_i$  uptake. In high  $P_i$  medium, PhoU2 serves as the master negative regulator of Pst2 transporter function and PnpR~P levels (post-transcriptionally).  $\Delta$ *phoU2* mutants have high PnpR~P levels and induction of the *pst1* operon, poor growth, and sensitivity to antibiotics, possibly due to high  $P_i$  accumulation. In low  $P_i$  medium, Pst2 is still active, but PnpR~P amount and *pst1* operon levels increase. Together, these results support a model in which pneumococcus maintains high  $P_i$  transport in high and low  $P_i$  conditions that is required for optimal capsule biosynthesis.

**Keywords:** PhoU, PnpRS two-component system (TCS), PnpR~P phosphorylation, Pst1 and Pst2  $P_i$  ABC transporters, NptA  $Na^+/P_i$  co-transporter

## INTRODUCTION

Phosphorus is an essential element in all cells because of its structural and metabolic roles in nearly all biological processes, including the composition of nucleic acids, phospholipids, and energy intermediates. A preferred source of phosphorous for bacterial cells is environmental inorganic orthophosphate ( $PO_4^-$ ;  $P_i$ ). The mechanism of extracellular  $P_i$  uptake has been studied intensively

in *Escherichia coli* and *Bacillus subtilis* as model organisms (Hulett, 1993; Takemaru et al., 1996; Wanner, 1996; Qi et al., 1997; Lamarche et al., 2008; Hsieh and Wanner, 2010; Botella et al., 2011, 2014), and recently in other bacterial species (Braibant et al., 1996; Gonin et al., 2000; Zaborina et al., 2008; Rifat et al., 2009; Shi and Zhang, 2010; Burut-Archanai et al., 2011; Cheng et al., 2012; Wang et al., 2013; de Almeida et al., 2015; Lubin et al., 2016). Generally, bacterial high-affinity  $P_i$  uptake systems consist of an ATP-binding cassette (ABC) transporter, designated as Pst (for phosphate-specific transporter), which contains at least four component subunits: an extracellular  $P_i$  binding protein (PstS), two transmembrane channel proteins (PstCA), and a cytoplasmic ATPase (PstB) (see **Figure 1**; Hsieh and Wanner, 2010). The expression of most bacterial Pst transporters is regulated at the transcriptional level by a two-component regulatory system (TCS), which has different designations in different bacteria (Hulett, 1993; Novak et al., 1999; Throup et al., 2000; Howell et al., 2006; Glover et al., 2007). Many bacteria also regulate  $P_i$  uptake by an ancillary negative regulatory protein, designated PhoU (Steed and Wanner, 1993; Botella et al., 2011, 2014; de Almeida et al., 2015; Lubin et al., 2016).

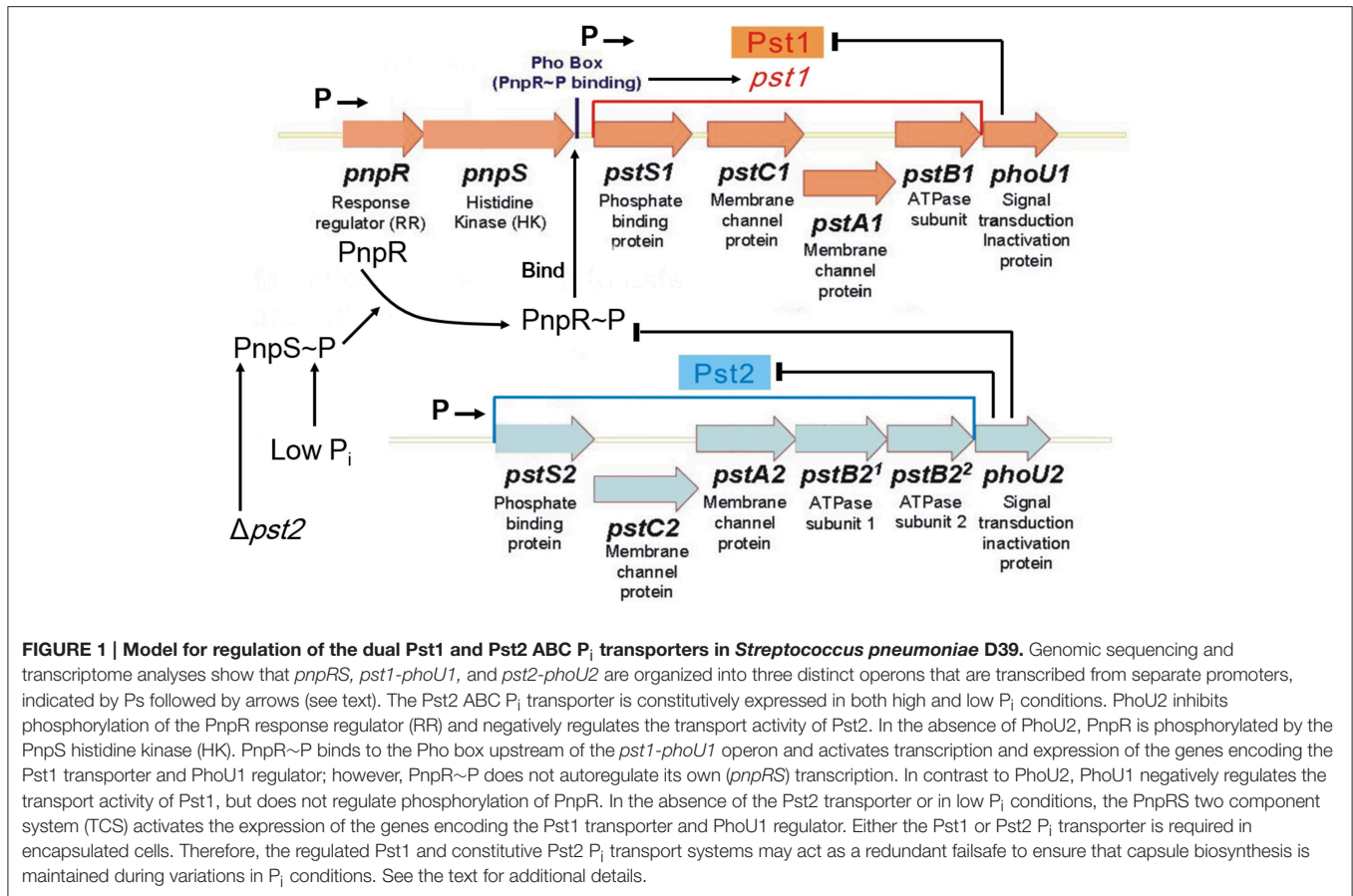
In *E. coli* and related enterobacteria, the histidine kinase (HK) and response regulator (RR) that mediate  $P_i$  transport are designated as PhoR and PhoB, respectively, and the regulation of  $P_i$  uptake involves a PhoU regulator (Hsieh and Wanner, 2010; Gardner et al., 2014, 2015). Briefly, when  $[P_i] > 4.0 \mu\text{M}$ , the expression of the *phoB-phoR* regulator and *pst* transporter operons is inhibited by PhoU by a mechanism described below (Hsieh and Wanner, 2010). When the  $[P_i]$  is depleted to  $<4.0 \mu\text{M}$ , PhoU releases inhibition of the PhoR HK and the PstB subunit of the transporter, allowing autophosphorylation of the PhoR HK, phosphoryl transfer to the PhoB RR, and activation of transcription by PhoB~P of operons in the phosphate (*pho*) regulon, including the *pst* transporter, the *phoB-phoR* regulator, and other operons involved in the uptake and assimilation of phosphorous-containing compounds (Wanner, 1996; Hsieh and Wanner, 2010). PhoB~P activates transcription by binding to the Pho box sequence upstream from the promoters of the regulon operons, including *phoB-phoR*, which provides autoregulation of the TCS proteins (Wanner, 1996; Martin, 2004; Lubin et al., 2016). Usually, the sequence of the consensus Pho box is different between Gram-negative (e.g., *E. coli*) and Gram-positive (e.g., *B. subtilis*) bacteria (Martin, 2004). Since the Pst transporter is not activated in *E. coli* at high  $P_i$  concentrations, this system is considered as a high-affinity transporter that works predominantly at low  $P_i$  concentrations (Wanner, 1996). PhoB/R, the Pst transporter, and members of the Pho regulon are important for virulence in many pathogenic Gram-negative bacteria, including *E. coli*, *Vibrio cholerae*, *Proteus mirabilis*, and *Pseudomonas aeruginosa* (Jacobsen et al., 2008; Lamarche et al., 2008; Zaborina et al., 2008; Pratt et al., 2010; Chekabab et al., 2014a,b). In *P. aeruginosa*, the PstS  $P_i$  binding protein also plays roles in adhesion and a  $P_i$ -independent role in biofilm formation (Zaborina et al., 2008; Neznansky et al., 2014; Shah et al., 2014).

PhoU is a negative regulator of Pho regulon expression in *E. coli* and many other bacteria (Muda et al., 1992; Wanner,

1996; Hsieh and Wanner, 2010; Gardner et al., 2014; de Almeida et al., 2015). Although, PhoU is an important regulator in many bacteria, it is notably absent from certain Gram-positive bacteria, including *B. subtilis* (Qi et al., 1997; Moreno-Letelier et al., 2011). *phoU* deletion in *E. coli*, *P. aeruginosa*, and *Mycobacterium marinum* leads to growth defects (Steed and Wanner, 1993; Wanner, 1996; Rice et al., 2009; Wang et al., 2013; de Almeida et al., 2015). In *E. coli*, this growth defect is reversed by deletion of the *pst* transporter operon or the *phoBR* TCS operon (Steed and Wanner, 1993; Wanner, 1996). These observations suggest that the growth defect of *phoU* mutants is caused by unregulated function of the Pst transport system, leading to excess  $P_i$  accumulation (Wanner, 1996; Rice et al., 2009).  $\Delta phoU$  mutants also accumulate increased amounts of poly-orthophosphate (poly- $P_i$ ) in *E. coli*, *M. marinum*, *P. aeruginosa*, and *Caulobacter crescentus* (Morohoshi et al., 2002; Hirota et al., 2013; Wang et al., 2013; de Almeida et al., 2015; Lubin et al., 2016). Poly- $P_i$  also accumulates in *E. coli* K-12 cells in stationary phase in high  $P_i$  medium, and this accumulation is correlated with inhibition of biofilm formation mediated by PhoB~P RR phosphorylation with acetyl-phosphate acting as donor (Grillo-Puertas et al., 2016). The rate of  $P_i$  uptake was reported to increase in an *E. coli*  $\Delta phoU$  in one study (Rice et al., 2009), but not in another (Steed and Wanner, 1993). Besides defective growth,  $\Delta phoU$  mutants exhibit higher sensitivity to a diverse range of antibiotics in *E. coli*, *Mycobacterium tuberculosis*, *M. marinum*, and *P. aeruginosa* (Li and Zhang, 2007; Shi and Zhang, 2010; Wang et al., 2013; de Almeida et al., 2015) and a defect in mutagenic DNA break repair in *E. coli* (Gibson et al., 2015).

Recent papers demonstrate that *E. coli* PhoU interacts with the PAS domain of the PhoR HK and with the PstB ATPase protein, in support of the dual inhibition of Pho regulon transcription and Pst mediated transport in *E. coli* (Gardner et al., 2014, 2015). Three crystal structures of PhoU-like proteins have been reported from *Aquifex aeolicus*, *Thermotoga maritima*, and *P. aeruginosa* (Liu et al., 2005; Oganessian et al., 2005; Lee et al., 2014), showing that PhoU consists of two symmetric, three-alpha-helix bundles. However, these PhoU proteins showed several quaternary structures in crystals, including monomer, dimer, or hexamer packing (Liu et al., 2005; Oganessian et al., 2005; Lee et al., 2014). Gel filtration shows that purified *E. coli* PhoU forms a dimer in solution (Gardner et al., 2014). In addition, divalent cation binding of magnesium and manganese is required for *E. coli* PhoU binding to the cytoplasmic side of the inner membrane and may play a role in formation of a ternary regulatory complex containing PhoU, PhoR, and PstB (Gardner et al., 2014). On the other hand, a recent study suggests that a different paradigm operates in *C. crescentus*, where PhoU does not modulate PhoR HK activity directly (Lubin et al., 2016). Instead, PhoU may negatively regulate the activity of the Pst transporter in response to  $P_i$  availability in *C. crescentus* (Lubin et al., 2016).

Besides Pst ABC transporters,  $P_i$  is taken up by symporter secondary transport systems. In *E. coli*, two additional  $P_i$  transporters, PitA and PitB, have been identified that are symporters of divalent cations, such as  $\text{Mg}^{2+}$  and  $\text{Ca}^{2+}$  (van Veen



et al., 1994; Wanner, 1996; Harris et al., 2001; Jackson et al., 2008). PitA and PitB have been considered as low-affinity  $P_i$  transporters that predominantly function in high  $P_i$  environments (Wanner, 1996; Hsieh and Wanner, 2010). Moreover, expression of *pitA* is induced by  $Zn^{2+}$  addition (Jackson et al., 2008), suggesting that PitA may act primarily as a metal ion transporter instead of a  $P_i$  transporter (Beard et al., 2000).

Unlike *E. coli*, *B. subtilis*, or *C. crescentus*, which contain only one Pst transporter, *Streptococcus pneumoniae* (pneumococcus) encodes two evolutionarily distinct  $P_i$  ABC pump transporters, Pst1 and Pst2 (Figure 1; Lanie et al., 2007; Moreno-Letelier et al., 2011). The multigene *pst1* and *pst2* operons are located at different locations in the pneumococcus chromosome (Lanie et al., 2007). Only one *phoBR*-like TCS, *pnpRS*, encoding the PnpR RR and the PnpS HK, is encoded upstream of the *pst1* operon, and both the *pst1* and *pst2* transporter operons encode separate PhoU-family regulators, designated PhoU1 and PhoU2 (Figure 1). The PnpRS TCS, Pst1 transporter, and PhoU1 were initially studied in unencapsulated *S. pneumoniae* laboratory strain R6x (Novak et al., 1999), before the discovery of the second Pst2 transporter and PhoU2 regulator. This work indicated that *pnpRS* operon expression was not regulated by  $P_i$  amount, and that mutants deficient in the PstB1 ATPase subunit seemed to show decreased  $P_i$  uptake in certain growth media (Novak et al., 1999). Subsequent work suggests that upregulation of

*pst1* operon expression is correlated with increased  $\beta$ -lactam antibiotic resistance in low-affinity *pbp2x* mutants and some clinical isolates of *S. pneumoniae* (Soualhine et al., 2005; Engel et al., 2014).

*S. pneumoniae* is a common commensal bacterium that primarily colonizes the human nasopharynx (Donkor, 2013; Chao et al., 2014; Hakansson et al., 2015; Short and Diavatopoulos, 2015), but can become an opportunistic pathogen, causing several serious respiratory and invasive diseases (Henriques-Normark and Tuomanen, 2013; Vernatter and Pirofski, 2013; Ferreira and Gordon, 2015; Gratz et al., 2015; Oliver and Swords, 2015). Therefore, the Pst1 and Pst2 transporters must mediate  $P_i$  acquisition from several niches with vastly different  $P_i$  concentrations in human hosts (Orihuela et al., 2001; Wilson, 2005, 2008). Signature-tagged mutagenesis (STM) screens and a study of the role of the PnpR RR (also called RR04) indicated that the *pnpRS*, *pst1*, and *pst2* operons are all required for full pneumococcal virulence (Polissi et al., 1998; Throup et al., 2000; Hava and Camilli, 2002; McCluskey et al., 2004; Paterson et al., 2006; Trihn et al., 2013). Consistent with these earlier studies, a recent Tn-Seq study showed that PhoU1 is important for nasopharynx colonization, whereas PhoU2 is important for lung infection (van Opijnen and Camilli, 2012).

In this report, we studied the transcriptional and functional regulation of the pneumococcal Pst1 and Pst2  $P_i$  transporters

under growth conditions containing high ( $\approx 18$  mM) or low ( $\approx 100$   $\mu$ M) concentrations of  $P_i$ . Our results show that *pst2* operon transcription is constitutive, but Pst2 transporter activity is negatively regulated by PhoU2. In addition, PhoU2 negatively regulates PnpR RR phosphorylation and transcription of the *pst1* operon at high concentrations of  $P_i$ . Therefore, PhoU2 resembles *E. coli* PhoU in that it functions in regulating the level of RR phosphorylation, besides modulating Pst2 transporter activity. In contrast, PhoU1 resembles *C. crescentus* PhoU in that it only regulates Pst1 transporter activity and does not modulate PnpRS TCS function. Our results also indicate that encapsulated *S. pneumoniae* requires the function of Pst1 or Pst2 for growth, whereas a symporter, named NptA, can provide sufficient  $P_i$  to allow the growth of unencapsulated mutants in high  $P_i$  conditions.

## MATERIALS AND METHODS

### Bacterial Strains and Growth Conditions

Strains used in this study are listed in Table S1. Encapsulated strains were derived from parent strain IU1781 (D39 *cps*<sup>+</sup> *rpsL1*), and unencapsulated strains were derived from parent strains IU1945 (D39  $\Delta$ *cps*), IU1824 (D39  $\Delta$ *cps* *rpsL1*), and IU3309 (D39  $\Delta$ *cps2E* *rpsL1*), which are derivatives of serotype 2 *S. pneumoniae* strain D39 IU1690 (Lanie et al., 2007). Strains containing antibiotic markers were constructed by transformation of competent pneumococcal cells with linear DNA amplicons synthesized by overlapping fusion PCR (Ramos-Montanez et al., 2008; Tsui et al., 2010). Strains containing markerless alleles in native chromosomal loci were constructed using allele replacement via the Pc-[*kan-rpsL*<sup>+</sup>] (Janus cassette; Sung et al., 2001). Primers used to synthesize different amplicons are listed in Table S2. All constructs were confirmed by DNA sequencing of chromosomal regions corresponding to the amplicon region used for transformation. Bacteria were grown on plates containing trypticase soy agar II (modified; Becton-Dickinson) and 5% (vol/vol) defibrinated sheep blood (TSII-BA). Plates were incubated at 37°C in an atmosphere of 5% CO<sub>2</sub>. For selections of transformants, TSII-BA plates contained 250  $\mu$ g/mL kanamycin, 0.3  $\mu$ g/mL erythromycin, or 250  $\mu$ g/mL streptomycin. Strains were cultured statically in Becton-Dickinson brain heart infusion (BHI) broth at 37°C in an atmosphere of 5% CO<sub>2</sub>, and growth was monitored by OD<sub>620</sub> as described before (Tsui et al., 2016). Transformants were single-colony-isolated on TSII-BA plates containing antibiotics twice before growth in antibiotic-containing BHI broth for storage (Tsui et al., 2016). All mutant constructs were confirmed by DNA sequencing of chromosomal regions corresponding to the amplicon region used for transformation.

Static growth in BHI broth, which contains  $\approx 18$  mM  $P_i$ , was used as a high  $P_i$  condition. For growth curves, strains were inoculated into 3 mL of BHI broth, serially diluted, and grown overnight. The next day, cultures with OD<sub>620</sub> = 0.1–0.3 were diluted into 5 mL of fresh BHI broth to OD<sub>620</sub>  $\approx 0.002$ , and growth was monitored hourly. C+Y medium (Lacks and Hotchkiss, 1960) was used for studies of moderately low  $P_i$

condition. We determined that C+Y broth (no added  $P_i$ ) already contains  $\approx 1.5$  mM  $P_i$  (see Results). A modified chemically defined medium (mCDM) (Carvalho et al., 2013) was used for moderate and low  $P_i$  conditions. To optimize growth, the concentrations of choline-HCl and all amino acids amounts were increased by 1000-fold and tyrosine was added to 100 mg/L compared to the CDM recipe in Carvalho et al. (2013). In addition, 40 mM MOPS buffer was added to mCDM, which was adjusted to a final pH = 7.4 with 10 M NaOH. mCDM contains 36.4 mM  $P_i$  (Carvalho et al., 2013). mCDM with no  $P_i$  was made by omitting KH<sub>2</sub>PO<sub>4</sub> and K<sub>2</sub>HPO<sub>4</sub> and adding KCl to 50.8 mM. mCDM media with 2 mM or 1 mM  $P_i$  was made by mixing mCDM and mCDM lacking  $P_i$  in a ratio of 2–34.4 or 1–35.4, respectively. mCDM with 1 mM  $P_i$  was diluted 10 or 100-fold with mCDM lacking  $P_i$  to make mCDM with 100  $\mu$ M and 10  $\mu$ M  $P_i$ , respectively. For growth in mCDM, 3 mL overnight cultures were grown as described above, and the next day, cultures with OD<sub>620</sub> = 0.1–0.3 were centrifuged (5125  $\times$  g, 5 min, 25°C), washed with 3 mL mCDM lacking  $P_i$  twice, and resuspended in 3 mL mCDM lacking  $P_i$ . Cells were then diluted in 5 mL of mCDM with 2, 1 mM, 100, 10  $\mu$ M, or no  $P_i$  to OD $\approx 0.005$  and growth of static cultures in an atmosphere of 5% CO<sub>2</sub> was monitored hourly at OD<sub>620</sub>.

### Antibiotic Disk Diffusion Assays

Overnight cultures were diluted and grown in 5 mL of BHI to OD<sub>620</sub>  $\approx 0.1$ . 100  $\mu$ L of cultures were mixed with 3 mL of nutrient-broth soft agar [0.8% (w/v) nutrient broth and 0.7% (w/v) Bacto Agar (Difco)] and poured onto TSAII-BA plates. After 15 min, antibiotics disks were placed at the middle of plates, which were incubated at 37°C in an atmosphere of 5% CO<sub>2</sub> overnight for 16 h. Diameters of zones of growth inhibition were measured with a ruler, and *P*-values were calculated by unpaired *t*-test in GraphPad Prism. Antibiotic disks were from Becton, Dickinson Co.: cefotaxime (30  $\mu$ g); cefazolin (30  $\mu$ g); cefamandole (30  $\mu$ g); ceftazidime (30  $\mu$ g); amdinocillin (10  $\mu$ g); vancomycin (30  $\mu$ g); gentamicin (120  $\mu$ g); and tetracycline (30  $\mu$ g).

### RNA Preparation, qRT-PCR, and RNA-Seq Analyses

To study high  $P_i$  conditions, overnight cultures were diluted and grown in 5 mL of BHI to OD  $\approx 0.15$ . Cells were collected by centrifuging at 16,000  $\times$  g for 5 min at 4°C. 1 mL of RNAPro solution (MP Biomedicals) was added to resuspend cell pellets. The suspension was transferred to a Lysing Matrix B tube (MP Biomedicals), which was shaken 3X in FastPrep homogenizer (6.0 M/s for 40 s each). Cell debris and lysing matrix were removed by centrifugation at 16,000  $\times$  g for 5 min at 4°C. 700  $\mu$ L of supernates was transferred to a new microcentrifuge tube and incubated at room temperature for 5 min. 300  $\mu$ L of chloroform was then added followed by incubation at room temperature for 5 min. Mixtures were centrifuged at 16,000  $\times$  g for 5 min at 4°C. 280  $\mu$ L of the upper, aqueous phase was collected and mixed with 140  $\mu$ L of 100% Ethanol in a new microcentrifuge tube for RNA precipitation. RNA purification was done using miRNeasy minikit (Qiagen), including on-column treatment



with DNase I (Qiagen), following the manufacturer's instructions. 5  $\mu$ g of purified RNA was treated by DNase from a DNA-free DNA removal kit (Ambion). 125 ng of treated RNA was used to synthesize cDNA by a qScript Felex cDNA synthesis kit (Quanta Biosciences). Synthesized cDNA was diluted 1:6 in water and then serially diluted 1:5 in water three more times. qRT-PCR reactions contained 10  $\mu$ L of 2  $\times$  Brilliant III Ultra-Fast SYBR Green QPCR Master Mix (Agilent), 2  $\mu$ L of each 2  $\mu$ M primers (Table S3), 0.3  $\mu$ L of a 1:500 dilution of ROX reference dye, and 6  $\mu$ L of diluted cDNA. Samples were run in an MX3000P thermocycler (Stratagene) with Program MxPro v. 3.0. Transcript amounts were normalized to *gyrA* mRNA amount and compared with transcript amounts of the wild-type parent strain by unpaired *t*-test in GraphPad Prism (Kazmierczak et al., 2009).

To study low  $P_i$  conditions, bacteria from overnight BHI broth cultures were washed, diluted, and grown in 5 mL of mCDM medium containing 36.4 mM  $P_i$  to  $OD_{620} \approx 0.15$ . Cells were collected by centrifugation, washed twice with 5 mL mCDM lacking  $P_i$ , and resuspended in mCDM containing 36.4 mM  $P_i$  or 10  $\mu$ M  $P_i$ . Cultures were incubated at 37°C in an atmosphere of 5%  $CO_2$  for 30 min. Lysis, RNA extraction, purification, and qRT-PCR reactions were performed as described above, except that 16S rRNA was used to normalize transcript amounts, because *gyrA* was down-regulated under low  $P_i$  condition. All transcript amounts were compared with the wild-type parent strain grown in mCDM containing 36.4 mM.

RNA samples for RNA-Seq analyses were prepared from 30 mL cultures as described previously (Hoover et al., 2015) cDNA library construction, single-end, 100 bp-sequencing on a HiSeq 2000 sequencer (Illumina), and bioinformatic analyses were performed as described in Hoover et al. (2015). False-discovery rates (FDR) were calculated using Benjamini and Hochberg's algorithm (Benjamini and Hochberg, 1995) and a gene or region was defined as differentially expressed if it had an up- or down-fold change of 1.8 with a FDR < 0.05. RNA-Seq data were deposited in the NCBI GEO database under accession number GSE80637.

## Phos-Tag SDS-PAGE and Western Blot

Phos-tag SDS-PAGE and standard Western blotting were carried out as described previously (Wayne et al., 2010, 2012; Tsui et al., 2014). To study high  $P_i$  conditions, overnight BHI broth cultures were diluted and grown up to  $OD_{620} \approx 0.2$  in 30 mL of BHI. Cells expressing PnpR-L-FLAG<sup>3</sup> were lysed using a FastPrep homogenizer, and cell lysates were resolved by Phos-tag SDS-PAGE at 4°C (Wayne et al., 2012). Cells expressing PstS2-HA were lysed by the same method, but resolved by standard SDS-PAGE (Tsui et al., 2014). PnpR-L-FLAG<sup>3</sup> and PstS2-HA were detected by Western blotting as described previously (Tsui et al., 2014) using anti-FLAG or anti-HA antibody as primary antibody. Chemiluminescent signal in protein bands was quantitated by using an IVIS imaging system as described in Wayne et al. (2010).

To study low  $P_i$  conditions, strains were grown in 30 mL of mCDM to  $OD_{620} \approx 0.2$  as described above. Cells were collected by centrifugation and washed twice with 30 mL of mCDM lacking

$P_i$ . Cell pellets were resuspended in 30 mL of mCDM containing 36.4 mM  $P_i$  or lacking  $P_i$  and incubated statically at 37°C in an atmosphere of 5%  $CO_2$  for 40 min. Proteins samples were extracted and Phos-tag SDS-PAGE was performed as described above.

## Qualitative Quellung Assay for Capsule

Overnight cultures were diluted and grown in 5 mL of BHI to  $OD \approx 0.15$ . 1  $\mu$ L of culture was mixed with 1  $\mu$ L of Type 2 pneumococcal antiserum (Statens Serum Institut) on a glass slide. A cover slip was placed on top of the mixture, which was viewed immediately with a 100X objective by a phase-contrast microscope. Cells surrounded by capsule appear enlarged or swollen.

## $P_i$ Concentration Determination

The  $P_i$  amount in C+Y broth was determined by the colorimetric method described in Katewa and Katyare (2003). Briefly, standards were prepared by dilution of a  $KH_2PO_4$  stock to give final  $P_i$  concentrations of 2.5, 5.0, 10.0, 20.0, and 40.0  $\mu$ M  $P_i$  in 2.4 mL of water in glass tubes. 0.8 mL of 3N  $H_2SO_4$  was added to each standard tube. 0.4 mL of 2.5% (w/v) ammonium molybdate (prepared in 3N  $H_2SO_4$ ) was added in each tube. Last, 0.4 mL of reducing agents (20 mg of hydrazine sulfate and 20 mg of ascorbic acid dissolved in 1 mL of 0.1N  $H_2SO_4$ ) was added to each tube. After 2 h at room temperature,  $A_{820}$  was determined and plotted to generate a standard curve. C+Y broth (no added  $P_i$ ) was diluted 100X with water,  $P_i$  content was assayed as described above, and  $P_i$  concentration was determined from the standard curve.

## $P_i$ Uptake Assays

To study high  $P_i$  conditions, strains were grown in 5 mL of BHI broth to  $OD_{600} \approx 0.2$ . Cells were centrifuged at  $5125 \times g$  for 5 min at room temperature, washed twice with 5 mL of mCDM lacking  $P_i$ , and resuspended at room temperature in 5 mL of mCDM lacking  $P_i$ .  $K_2H^{32}PO_4$  ( $10^7$ – $10^8$  dpm; 8500–9120 Ci/mmol; Perkin Elmer) was added to a final concentration of 1 mM at  $t=0$ , and 100  $\mu$ L of cells was collected by vacuum filtration (0.22  $\mu$ m GSWP; 13 mm diameter; Millipore) at 1, 2, 4, 6, 10 min after addition of  $^{32}P$ . Filters were washed 3X with 3 mL of room-temperature 1  $\times$  PBS (Ambion). Washed filters were transferred individually into 20-mL glass scintillation vials to which 5 mL of a biodegradable counting cocktail was added. Dpm of each sample was determined using a TRI-CARB 2100TR Liquid Scintillation Counter (Perkin Elmer),  $P_i$  amount incorporated at each time point was calculated.

To study low  $P_i$  condition, strains were grown to  $OD_{600} \approx 0.2$  in mCDM containing 36.4 mM  $P_i$  as described above. Cells were centrifuged at  $5125 \times g$  for 5 min at room temperature, and washed twice with 5 mL of mCDM lacking  $P_i$ . Cell pellets were resuspended in 5 mL of mCDM lacking  $P_i$  and incubated 1 h at 37°C in an atmosphere of 5%  $CO_2$ . 200  $\mu$ M of  $K_2H^{32}PO_4$  ( $10^7$ – $10^8$  dpm) was added at  $t = 0$ , and samples were withdrawn, filtered, and counted as described above.

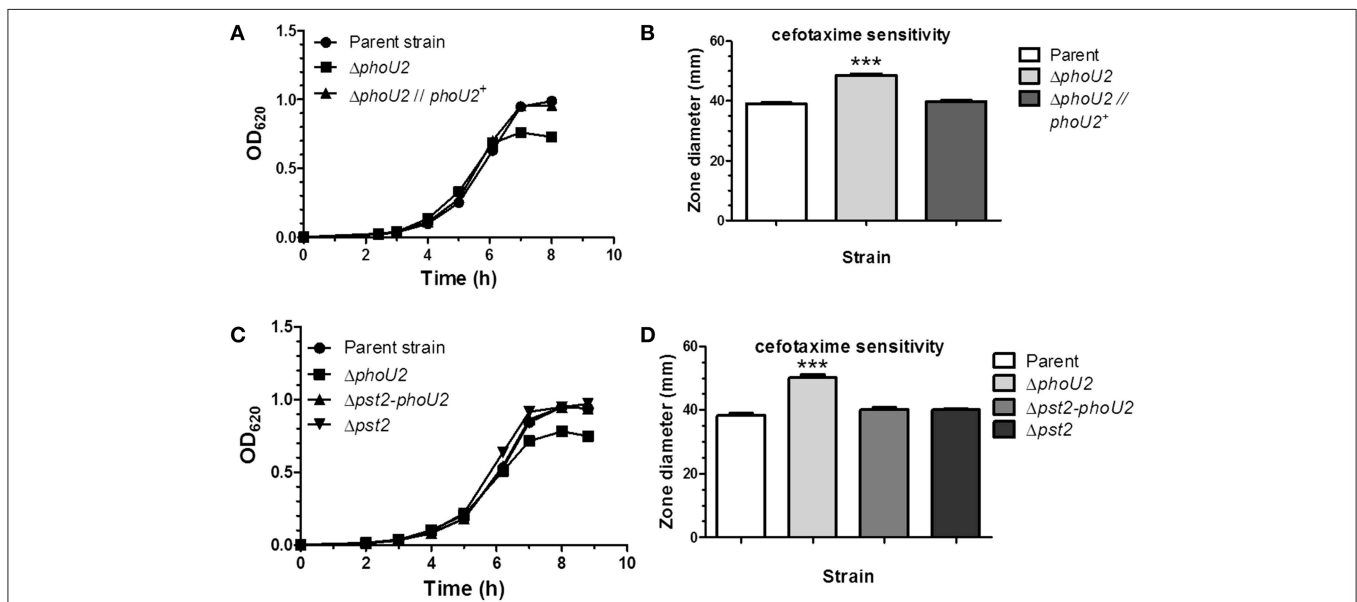
## RESULTS

### $\Delta phoU2$ Mutants Show a Growth Defect and Increased Sensitivity to a Range of Antibiotics That is Reversed by Inactivation of Pst2 Transport

The Pst transporter is not needed for growth of *E. coli* at high  $P_i$  concentrations  $>4\mu\text{M}$ , and  $\Delta phoU$  mutations lead to a severe growth defect that is reversed by inactivation of the Pst transporter (Steed and Wanner, 1993; Rice et al., 2009). To determine the roles of the Pst1 and Pst2 transporters and their regulation, we constructed a series of markerless deletion mutants [ $\Delta phoU2$  (IU6375);  $\Delta phoU1$  (IU6377);  $\Delta pnpRS$  (IU6381);  $\Delta pst2$  (IU6610);  $\Delta pst1$  (IU6638)] in *S. pneumoniae* serotype 2 strain D39, which is encapsulated and virulent (Lanie et al., 2007; Figure 1, Table S1). Mutants were first grown in BHI broth, which contains a high  $P_i$  concentration ( $\approx 18\text{ mM}$ ). Only the  $\Delta phoU2$  mutant showed a significant decrease in growth yield compared to the parent and other mutants (Figure 2A, Figure S1A, Table S4). Growth yield was restored when the  $\Delta phoU2$  mutation was complemented by an ectopic copy of the  $phoU2^+$  gene expressed from the  $P_{ftsA}$  promoter at the  $bgaA$  site (IU6397) (Figure 2A, Table S4). Similar results were obtained in mutants in unencapsulated D39 derivative strain K579 and E579 (data not shown).

Inactivation of  $phoU$  in *E. coli* generates higher sensitivity to various kinds of antibiotics than the parent strain (Li and Zhang, 2007). Since the pneumococcal  $\Delta phoU2$  mutant shows a similar growth defect as the  $\Delta phoU$  mutant in *E. coli* (Figure 2A, Figure S1A), we tested antibiotic sensitivity to several antibiotics. Of the Pho regulon mutants tested in the encapsulated strain, only the  $\Delta phoU2$  mutant showed increased sensitivity to  $\beta$ -lactams and other classes of antibiotics, including glycopeptides and protein synthesis inhibitors, on plates that contain relatively high  $P_i$  content (Figure 2B, Figure S1B, and Table S5).

Previous work in *E. coli* (Steed and Wanner, 1993; Wanner, 1996; Rice et al., 2009; Hsieh and Wanner, 2010) and *C. crescentus*, in which PhoU is essential for growth (Lubin et al., 2016), indicated that the reduced growth of  $phoU$  deletion or depletion mutants could be reversed by inactivation of the Pst pump. This reversal was interpreted to mean that PhoU negatively regulates the Pst transporter itself, and in its absence, deleterious phosphate compounds accumulate that disrupt growth and metabolism. In the pneumococcal encapsulated D39 genetic background, a  $\Delta pst2$ - $phoU2$  deletion mutant lacking the Pst2 transporter and PhoU2 regulator grew similar to the parent strain and did not show increased sensitivity to antibiotics caused by the absence of PhoU2 alone (Figures 2C,D, Table S4). Likewise, single deletions of genes encoding each component of the Pst2 transporter restored growth yield of a  $\Delta phoU2$



**FIGURE 2 | Deletion of  $phoU2$  leads to a lower growth yield and increased  $\beta$ -lactam antibiotic sensitivity that are reversed by a  $\Delta pst2$  mutation. (A)** Representative growth curves in BHI broth ( $\approx 18\text{ mM } P_i$ ) of encapsulated parent strain (IU1781), a  $\Delta phoU2$  mutant (IU6375), and a  $\Delta phoU2$  mutant complemented by ectopic expression of PhoU2<sup>+</sup> (IU6397). Strains were grown as described in Materials and Methods. A linear scale for OD<sub>620</sub> is used to emphasize differences in growth yields. Growth yields and rates are quantitated for multiple determinations in Table S4. **(B)** Cefotaxime sensitivity assays of encapsulated parent strain (IU1781), a  $\Delta phoU2$  mutant (IU6375), and a PhoU2<sup>+</sup>-complemented  $\Delta phoU2$  mutant (IU6397). Cefotaxime disk sensitivity assays of bacteria grown in BHI broth were performed as described in Material and Methods. *P*-values were calculated by unpaired *t*-tests relative to the parent strain using GraphPad Prism; ( $n \geq 3$ ); \*\*\**P* < 0.001. Increased sensitivity to other  $\beta$ -lactam antibiotics, vancomycin, gentamicin, and tetracycline of a  $\Delta phoU2$  mutant compared to its isogenic parent strain is shown in Table S5. **(C)** Representative growth curves of encapsulated parent strain (IU1781) and  $\Delta phoU2$  (IU6375),  $\Delta pst2$ - $phoU2$  (IU6550), and  $\Delta pst2$  (IU6610) mutants in BHI broth. **(D)** Cefotaxime sensitivity assays for encapsulated parent strain (IU1781), and  $\Delta phoU2$  (IU6375),  $\Delta pst2$ - $phoU2$  (IU6550), and  $\Delta pst2$  (IU6610) mutants. ( $n \geq 3$ ); \*\*\**P* < 0.001.

mutant back to wild-type (data not shown). Western blot analysis showed that the  $\Delta phoU2$  mutation did not change the cellular amount of the PstS2 transporter subunit fused to the HA epitope tag (Figure S3), and by inference the amount of the Pst2 transporter. We conclude that the decreased growth yield and antibiotic sensitivity of encapsulated pneumococcus  $\Delta phoU2$  mutants in high  $P_i$  conditions are dependent on function of the Pst2 transporter, consistent with negative regulation of the Pst2 transporter by PhoU2.

## PhoU2 Negatively Regulates Transcription Activation of the *pst1* Operon by the PnpRS TCS in High $P_i$ Conditions

As noted in the Introduction, PhoU negatively regulates the PhoBR TCS in *E. coli*, but not in *C. crescentus* (Hsieh and Wanner, 2010; Lubin et al., 2016). Consequently, we tested whether  $\Delta phoU1$  or  $\Delta phoU2$  deletions affected transcript levels of the *pst1* or *pst2* operons under high  $P_i$  conditions (see Figure 1). We first performed RNA-Seq analyses of  $\Delta phoU2::kanrpsL^+$  and  $\Delta phoU2::kanrpsL^+ \Delta phoU1::P_c-erm$  mutants growing in early-middle exponential phase in BHI broth, which contains a high (18 mM) concentration of  $P_i$  (Table S6). In both strains, only transcript amounts of the *pst1* transporter operon, including *phoU1* in the single mutant, were strongly induced ( $\approx 22X$ ). The transcript amounts of the *pnpRS* regulator and *pst2* transporter operons were not induced, and the number of other genes in the Pho regulon appears to be limited in *S. pneumoniae* D39 (Table S6). Notably, pneumococcus encodes neither an alkaline phosphatase (*phoA*)

nor a pathway for synthesis of teichuronic acids lacking phosphate (Wanner, 1996; Botella et al., 2011, 2014). Besides the strong induction of *pst1* operon transcription, there were small (2-4X) changes in the relative amounts of only a handful of other transcripts, including some corresponding to metabolic and stress-responsive genes, possibly reflecting the defective growth of these *phoU2* mutants. In both mutants, one of the stronger responses was a decrease in the relative transcript amounts of the genes encoding the glycerol facilitator (GlpF) and glycerol kinase (GlpK). A putative, somewhat degenerate Pho box is located -125 bp upstream of the *glpK* reading frame. Together, these results suggested that PhoU2 negatively regulates *pst1* operon expression under high  $P_i$  conditions, whereas the *pnpRS* and *pst2* operons are constitutively expressed.

These conclusions were confirmed by qRT-PCR analysis of combinations of markerless deletion mutations in the *pst* and *pnpRS* genes (Table 1). RNA-Seq transcriptome analysis indicates that the *pnpRS*, *pst1-phoU1*, and *pst2-phoU2* operons are separately transcribed (Table S6, Figure 1, Figures S2A,B). Hence, we quantitated the relative amounts of the *pnpR*, *pstS1*, and *pstS2* transcripts normalized to *gyrA* transcript amount by qRT-PCR to represent *pnpRS*, *pst1-phoU1*, and *pst2-phoU2* operon expression (see Materials and Methods; Wayne et al., 2012). Consistent with the RNA-Seq results, the  $\Delta phoU2$  mutations caused  $\approx 16X$  increase in *pst1-phoU1* operon transcript amount, but no change in expression of *pst2* or *pnpRS* operon (Table 1, line 2). In contrast, a  $\Delta phoU1$  mutation did not cause a significant change in the relative amounts of transcript from any of the three operons (Table 1, line 3). No increase in *pst1-phoU1* transcript

**TABLE 1 | Relative transcript amounts of the *pst1*, *pst2*, and *pnpRS* operons in regulatory mutants<sup>a</sup>.**

| Strains <sup>b</sup>                          | Relative transcript amount of <i>pstS1</i> <sup>c</sup> | Relative transcript amount of <i>pstS2</i> <sup>d</sup> | Relative transcript amount of <i>pnpR</i> <sup>e</sup> |
|---|---|---|--|
| 1. Parent strain (IU1781)                     | $\equiv 1$  | $\equiv 1$  | $\equiv 1$   |
| 2. $\Delta phoU2$ (IU6375)                    | $+16.0 \pm 1.8$ ( $n = 4$ ) (***) <sup>f</sup>          | $+1.2 \pm 0.6$ ( $n = 2$ ) (ns)                         | $-1.2$ ( $n = 1$ )                                     |
| 3. $\Delta phoU1$ (IU6377)                    | $+1.6 \pm 0.4$ ( $n = 4$ ) (ns)                         | $+1.3 \pm 0.4$ ( $n = 2$ ) (ns)                         | $+1.1$ ( $n = 1$ )                                     |
| 4. $\Delta phoU2::phoU2^+$ (IU6397)           | $+1.3 \pm 0.3$ ( $n = 3$ ) (ns)                         | ND <sup>g</sup>   | ND   |
| 4. $\Delta pnpR$ (IU6379)                     | $-4.8 \pm 0.2$ ( $n = 2$ ) (**)                         | $-1.1 \pm 0.1$ ( $n = 2$ ) (ns)                         | ND   |
| 6. $\Delta pnpS$ (IU6496)                     | $-2.4 \pm 0.4$ ( $n = 2$ ) (**)                         | $-1.4 \pm 0.1$ ( $n = 2$ ) (ns)                         | ND   |
| 7. $\Delta pnpRS$ (IU6381)                    | $-2.0 \pm 0.6$ ( $n = 2$ ) (*)                          | $-1.1 \pm 0.2$ ( $n = 2$ ) (ns)                         | ND   |
| 8. $\Delta phoU2 \Delta pnpR$ (IU6573)        | $-4.4 \pm 1.1$ ( $n = 4$ ) (***)                        | ND  | ND   |
| 9. $\Delta phoU2 \Delta pnpS$ (IU6595)        | $-2.1 \pm 0.3$ ( $n = 4$ ) (**)                         | ND  | ND   |
| 10. $\Delta phoU2 \Delta pnpRS$ (IU6575)      | $-2.5 \pm 0.6$ ( $n = 5$ ) (**)                         | ND  | ND   |
| 11. $\Delta phoU2 \Delta phoU1$ (IU6499)      | $+19.6 \pm 1.6$ ( $n = 5$ ) (***)                       | $-1.4 \pm 0.1$ ( $n = 2$ ) (ns)                         | $+1.0$ ( $n = 1$ )                                     |
| 12. $\Delta pst2-phoU2$ (IU6550)              | $+43.8 \pm 4.2$ ( $n = 4$ ) (***)                       | –   | $+1.1 \pm 0.0$ ( $n = 3$ ) (ns)                        |
| 13. $\Delta pst2$ (IU6610)                    | $+44.8 \pm 1.5$ ( $n = 4$ ) (***)                       | –   | ND   |
| 14. $\Delta pst2-phoU2 \Delta phoU1$ (IU6612) | $+44.8 \pm 5.5$ ( $n = 3$ ) (**)                        | –   | ND   |
| 15. $\Delta pst1$ (IU6638)                    | –   | $+1.0$ ( $n = 1$ )                                      | ND   |

<sup>a</sup>RNA preparation and qRT-PCR were performed as described in Materials and Methods.

<sup>b</sup>Strains were markerless deletion mutants derived from encapsulated parent strain IU1781.

<sup>c</sup>Relative *pstS1* gene transcript amount was used to represent *pst1* operon expression.

<sup>d</sup>Relative *pstS2* gene transcript amount was used to represent *pst2* operon expression.

<sup>e</sup>Relative *pnpR* gene transcript amount was used to represent *pnpRS* operon expression.

<sup>f</sup>\*\*\* $P < 0.001$ ; \*\* $P < 0.01$ ; \* $P < 0.05$ ; ns, not significant.  $P$ -values were calculated by an unpaired  $t$ -test in GraphPad Prism.  $P$ -value is not available when  $n = 1$ .

<sup>g</sup>ND, not determined.

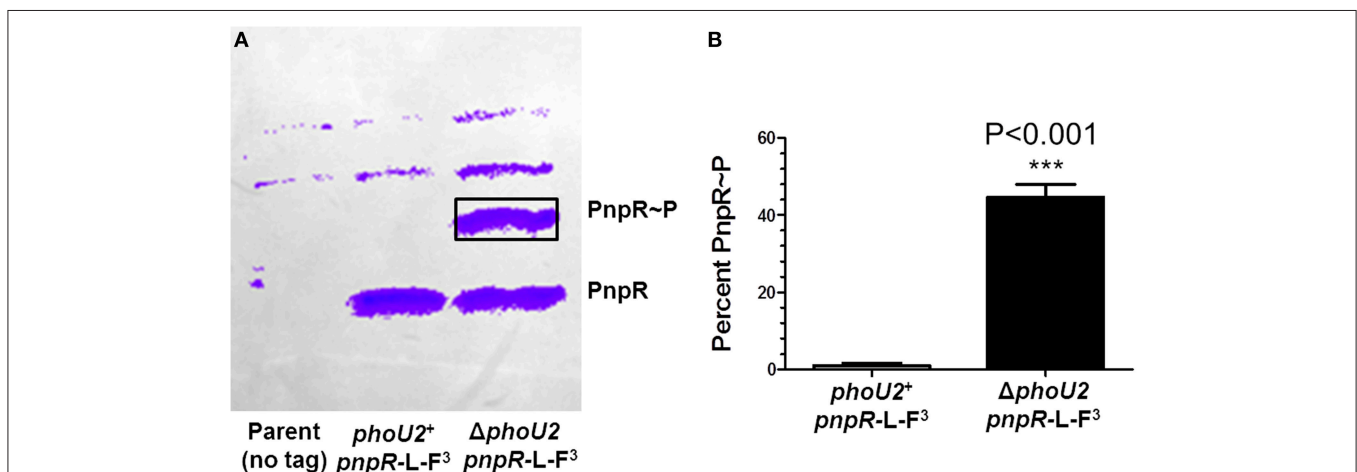
amount in the  $\Delta phoU2$  mutant was detected in complementation experiments in which a wild-type copy of  $phoU2^+$  was expressed from an ectopic site (Table 1, line 4).

We next determined that the increased transcription of the  $pst1-phoU1$  operon in the  $\Delta phoU2$  mutant is mediated by the PnpRS TCS through increased phosphorylation of the PnpR~P RR.  $\Delta pnpR$ ,  $\Delta pnpS$ , or  $\Delta pnpRS$  mutants showed slightly reduced  $pst1-phoU1$  operon transcript amounts compared to the parent strain, with no change in  $pst2-phoU2$  operon expression (Table 1, lines 5–7). Likewise,  $\Delta pnpR \Delta phoU2$ ,  $\Delta pnpS \Delta phoU2$ , and  $\Delta pnpRS \Delta phoU2$  double mutants showed reduced  $pst1-phoU1$  operon relative transcript amounts (Table 1, lines 8–10), instead of the sizable increase observed for the  $\Delta phoU2$  single mutant containing an active PnpRS TCS (Table 1, line 2). To confirm directly that PhoU2 acts as a negative regulator of PnpRS function under high  $P_i$  conditions, we performed Phos-tag SDS PAGE analysis to determine PnpR~P RR phosphorylation levels (see Materials and Methods; Figure 3). In these experiments, we fused three tandem copies of the FLAG epitope tag to the C-terminus of the PnpR RR regulator expressed from its normal chromosomal locus (Figure 3, Table S1). The PnpR-L-FLAG<sup>3</sup> RR induced  $pst1-phoU1$  transcription to a similar extent as wild-type (untagged) PnpR<sup>+</sup> in a  $\Delta phoU2$  mutant (data not shown). In the  $phoU2^+$  strain, essentially no PnpR~P (<1%) was detected in cells growing exponentially in high- $P_i$  BHI broth (Figure 3). In contrast, the  $\Delta phoU2$  mutant contained  $\approx 45\%$  PnpR~P, which accounts for the high ( $\approx 16X$ ) increase in  $pst1-phoU1$  transcript detected (Table 1, line 2). We conclude that PhoU2 negatively regulates PnpR~P amounts and transcription of the  $pst1-phoU1$  operon, but does not regulate transcription of the  $pnpRS$  or  $pst2-phoU2$  operon, which are constitutively expressed.

Consistent with this interpretation, a putative PnpR~P binding site (Pho-box) sequence (TTTACACAATCTTTACA; Martin, 2004) is located 92 bp upstream of  $pstS1$  reading frame gene (Figure 1), but no recognizable Pho-box sequences can be found upstream of the  $pnpRS$  or  $pst2-phoU2$  operon. Finally, we tested whether induction of  $pst1-phoU1$  operon expression contributes to the growth defect and antibiotic sensitivity of a  $\Delta phoU2$  mutant (Figure 2). A  $\Delta phoU2 \Delta pst1$  or  $\Delta phoU2 \Delta pst1-phoU1$  double mutant showed the same decrease in growth yield (Figure 4A, Table S4) and antibiotic sensitivity (Figure 4B) as the  $\Delta phoU2$  single mutant, indicating that these defects were caused primarily by misregulation of the Pst2 transporter in high  $P_i$  conditions, instead of induced expression of the Pst1 transporter.

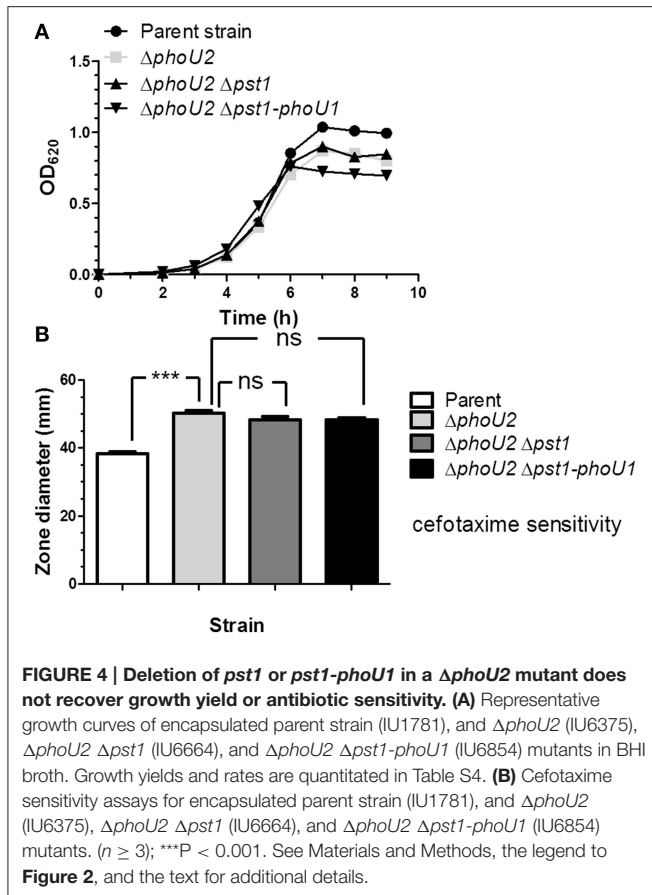
### Transcription of the $pst1$ Operon is Further Induced by the Absence of the Pst2 Transporter Under High $P_i$ Conditions

We next tested whether absence of the Pst2 transporter affects  $pst1$  operon expression in bacteria growing in high  $P_i$ -BHI broth. Surprisingly, relative transcript amounts from the  $pst1$  operon increased by  $\approx 44$  fold in the  $\Delta pst2 phoU2^+$ ,  $\Delta pst2-phoU2$ , and  $\Delta pst2-phoU2 \Delta phoU1$  mutant compared to the parent strain (Table 1, lines 12, 13, and 14). By contrast,  $pst2$  operon transcription is unchanged in a  $\Delta pst1 phoU1^+$  mutant compared to the parent (Table 1, line 15). The similar induction in the  $\Delta pst2 phoU2^+$  and  $\Delta pst2-phoU2$  mutants (Table 1, lines 12 and 13) implies that negative regulation of the PnpRS TCS by PhoU2 depends on a functional Pst2 transporter system. Moreover, the similarity of  $pst1$  induction in the  $\Delta pst2-phoU2 \Delta phoU1$  and other mutants implies that PhoU1 does not directly regulate  $pst1$  transcription. This conclusion was supported by the similar induction of  $pst1$  operon transcription

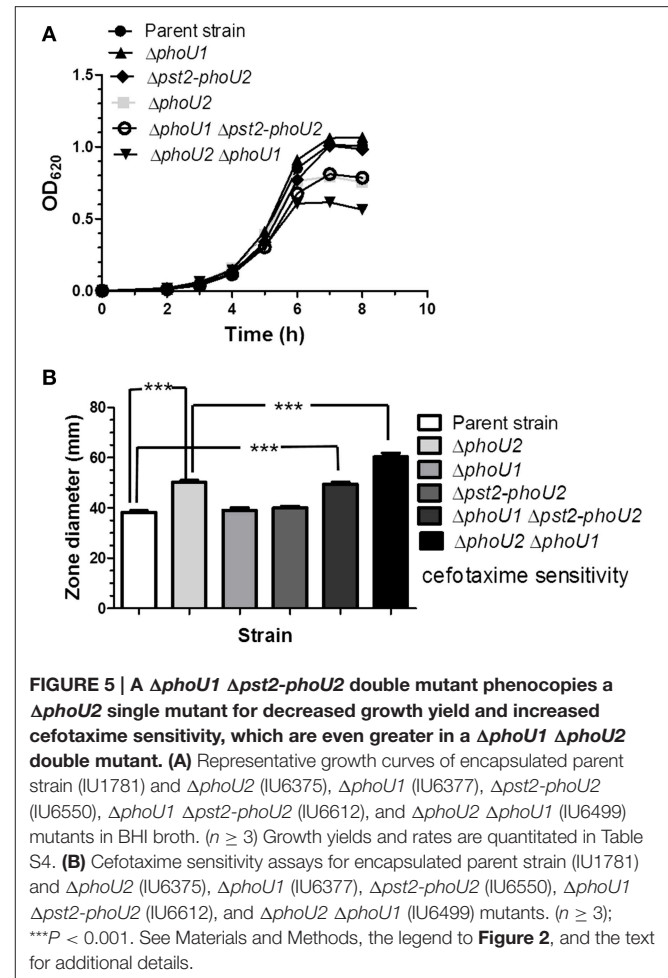


**FIGURE 3 | Approximately 45% of PnpR is phosphorylated (PnpR~P) in a  $\Delta phoU2$  mutant.** (A) Representative Phos-tag SDS-PAGE of the encapsulated parent strain not expressing a FLAG-tagged protein (IU1781, lane 1) and of the  $pnpR-L-FLAG^3$  (IU6689, lane 2), and  $\Delta phoU2 pnpR-L-FLAG^3$  (IU6687, lane 3) mutants growing exponentially in BHI broth. The gel was Western blotted with anti-FLAG antibody as described in Materials and Methods. The top two bands in all lanes are nonspecific. The upper anti-FLAG-specific band corresponds to phosphorylated PnpR~P, and the lower band corresponds to unphosphorylated PnpR. Control experiments show that the upper band is heat-sensitive, as expected for PnpR~P (Figure S4). (B) Quantification of 3 independent Phos-tag SDS-PAGE experiments. Less than 1% of PnpR was phosphorylated in the  $phoU2^+$  strain, whereas  $\approx 45\%$  of PnpR is phosphorylated (PnpR~P) in the  $\Delta phoU2$  mutant.  $P$ -value was determined by an unpaired  $t$ -test in GraphPad Prism; \*\*\* $P < 0.001$ .





**FIGURE 4 | Deletion of *pst1* or *pst1-phoU1* in a  $\Delta$ *phoU2* mutant does not recover growth yield or antibiotic sensitivity. (A)** Representative growth curves of encapsulated parent strain (IU1781), and  $\Delta$ *phoU2* (IU6375),  $\Delta$ *phoU2*  $\Delta$ *pst1* (IU6664), and  $\Delta$ *phoU2*  $\Delta$ *pst1-phoU1* (IU6854) mutants in BHI broth. Growth yields and rates are quantitated in Table S4. **(B)** Cefotaxime sensitivity assays for encapsulated parent strain (IU1781), and  $\Delta$ *phoU2* (IU6375),  $\Delta$ *phoU2*  $\Delta$ *pst1* (IU6664), and  $\Delta$ *phoU2*  $\Delta$ *pst1-phoU1* (IU6854) mutants. ( $n \geq 3$ ); \*\*\* $P < 0.001$ . See Materials and Methods, the legend to Figure 2, and the text for additional details.



**FIGURE 5 | A  $\Delta$ *phoU1*  $\Delta$ *pst2-phoU2* double mutant phenocopies a  $\Delta$ *phoU2* single mutant for decreased growth yield and increased cefotaxime sensitivity, which are even greater in a  $\Delta$ *phoU1*  $\Delta$ *phoU2* double mutant. (A)** Representative growth curves of encapsulated parent strain (IU1781) and  $\Delta$ *phoU2* (IU6375),  $\Delta$ *phoU1* (IU6377),  $\Delta$ *pst2-phoU2* (IU6550),  $\Delta$ *phoU1*  $\Delta$ *pst2-phoU2* (IU6612), and  $\Delta$ *phoU2*  $\Delta$ *phoU1* (IU6499) mutants in BHI broth. ( $n \geq 3$ ) Growth yields and rates are quantitated in Table S4. **(B)** Cefotaxime sensitivity assays for encapsulated parent strain (IU1781) and  $\Delta$ *phoU2* (IU6375),  $\Delta$ *phoU1* (IU6377),  $\Delta$ *pst2-phoU2* (IU6550),  $\Delta$ *phoU1*  $\Delta$ *pst2-phoU2* (IU6612), and  $\Delta$ *phoU2*  $\Delta$ *phoU1* (IU6499) mutants. ( $n \geq 3$ ); \*\*\* $P < 0.001$ . See Materials and Methods, the legend to Figure 2, and the text for additional details.

in the  $\Delta$ *phoU2* *phoU1*<sup>+</sup> and  $\Delta$ *phoU2*  $\Delta$ *phoU1* mutants (Table 1, lines 2 and 11). Instead, the growth characteristics of these mutants imply that PhoU1 negatively regulates the activity of the Pst1 transporter, in parallel to the negative regulation of the activity of Pst2 by PhoU2 (Figure 5A, Table S4). In BHI broth, the parent,  $\Delta$ *phoU1*, and  $\Delta$ *pst2-phoU2* mutant show similar growth and antibiotic sensitivity (Figure 5; Table S4). In contrast, the  $\Delta$ *phoU2* and  $\Delta$ *phoU1*  $\Delta$ *pst2-phoU2* mutants showed reduced growth yield and increased antibiotic sensitivity (Figure 5, Table S4), consistent with increased P<sub>i</sub> accumulation caused by misregulation of the Pst2 and Pst1 transporters, respectively. This interpretation was further supported by the reduced growth rate and yield and increased antibiotic sensitivity of the  $\Delta$ *phoU2*  $\Delta$ *phoU1* mutant compared to the  $\Delta$ *phoU2* and  $\Delta$ *pst2-phoU2*  $\Delta$ *phoU1* mutants (Figure 5; Table S4).

### Either the Pst1 or Pst2 Transporter is Required in the Encapsulated, But Not in the Unencapsulated, D39 Strain

*E. coli* encodes an alternate low-affinity P<sub>i</sub> uptake (Pit) system that functions in the absence of the high-affinity Pst transporter in high P<sub>i</sub> conditions (Wanner, 1996; Harris et al., 2001; Hsieh and Wanner, 2010). However, we were unable to construct  $\Delta$ *pst2*  $\Delta$ *pst1* mutants in the encapsulated strain of D39 (Table 2, top two sections), where the  $\Delta$ *nptA* amplicon was used as a positive

control in the transformations. Likewise, we were unable to construct a  $\Delta$ *pst2*  $\Delta$ *pnpRS* double mutant, where induction of the Pst1 transporter was negated by the absence of the PnpRS TCS (Table 1, top section; Figure 1). As expected, the *cps*<sup>+</sup>  $\Delta$ *pnpRS-pst1* mutant could be constructed (IU6133), where the Pst2 transporter is functional.

In all  $\Delta$ *pst2*  $\Delta$ *pst1* *cps*<sup>+</sup> transformations, several colonies (<10) appeared upon prolonged incubation (Table 2, top two sections). These suppressor colonies had a rougher appearance than the smooth colonies of D39 *cps*<sup>+</sup> strains (data not shown), suggesting that capsule production was lost or reduced in these  $\Delta$ *pst2*  $\Delta$ *pst1* transformants. The Quellung test for serotype 2 capsule (see Materials and Methods) confirmed that a suppressor strain (IU6413) had lost its capsule (data not shown). This result suggested that unlike in *cps*<sup>+</sup> strains, we would be able to construct  $\Delta$ *cps*  $\Delta$ *pst1*  $\Delta$ *pst2* mutants in the D39 genetic background. This hypothesis was confirmed by transformation experiments (Table 2, bottom two sections), and growth experiments showing that  $\Delta$ *cps*  $\Delta$ *pst1*  $\Delta$ *pst2* triple mutants grew comparably to the  $\Delta$ *cps* single mutant in BHI broth (Figure S5). Thus, we conclude that either the Pst1 or Pst2 P<sub>i</sub> transporter must be functional in encapsulated D39 strains and that the  $\Delta$ *pst1*  $\Delta$ *pst2* *cps*<sup>+</sup> double mutant is not viable.

**TABLE 2 | The  $\Delta pst1 \Delta pst2$  double mutant cannot be constructed in encapsulated strain D39<sup>a</sup>.**

| Recipient strain <sup>b</sup>                 | Amplicon <sup>c</sup>        | Number of colonies on transformation plates after $\approx 20$ h <sup>d</sup> |
|---|------------------------------|---|
| $\Delta pst2$ cps <sup>+</sup> (encapsulated) | $\Delta nptA$ (control)      | 100–150 ( $n = 3$ )   |
|   | $\Delta pnpRS$               | 0–10 ( $n = 3$ )  |
|   | $\Delta pst1$                | 0–4 ( $n = 3$ )   |
|   | $\Delta pst1$ - <i>phoU1</i> | 0 ( $n = 3$ )   |
|   | No DNA (control)             | 0 ( $n = 3$ )   |
| $\Delta pst1$ cps <sup>+</sup> (encapsulated) | $\Delta nptA$ (control)      | 100–150 ( $n = 3$ )   |
|   | $\Delta pst2$                | 0 ( $n = 3$ )   |
|   | $\Delta pst2$ - <i>phoU2</i> | 0 ( $n = 3$ )   |
|   | No DNA (control)             | 0 ( $n = 3$ )   |
| $\Delta pst2$ cps mutants (unencapsulated)    | $\Delta nptA$ (control)      | 100–150 ( $n = 3$ )   |
|   | $\Delta pnpRS$               | 250–300 ( $n = 3$ )   |
|   | $\Delta pst1$                | 100–150 ( $n = 3$ )   |
|   | $\Delta pst1$ - <i>phoU1</i> | 250–300 ( $n = 3$ )   |
|   | No DNA (control)             | 0 ( $n = 3$ )   |
| $\Delta pst1$ cps mutants (unencapsulated)    | $\Delta nptA$ (control)      | 100–150 ( $n = 3$ )   |
|   | $\Delta pst2$                | 100–150 ( $n = 3$ )   |
|   | $\Delta pst2$ - <i>phoU2</i> | 150–200 ( $n = 3$ )   |
|   | No DNA (control)             | 0 ( $n = 3$ )   |

<sup>a</sup> Transformations were performed as described in Materials and Methods.

<sup>b</sup> Transformations were performed into multiple cps<sup>+</sup> strains [IU1690 (D39); IU1781 (D39 rpsL1)] and cps mutants [IU1824 (D39  $\Delta cps$  rpsL1); IU1945 (D39  $\Delta cps$ ); IU3309 (D39  $\Delta cps2E$  rpsL1)] with similar results. D39  $\Delta cps2E$  rpsL1  $\Delta pst1 \Delta pst2$  mutants could not be repaired back to cps<sup>+</sup> (data not shown).

<sup>c</sup> Amplicons were synthesized as described in Materials and Methods (see Table S2). Amplicons used for transformations contained the P<sub>c</sub>-kanrpsL<sup>+</sup> or P<sub>c</sub>-erm antibiotic cassette for selection. Transformations with the  $\Delta nptA$  amplicon or without DNA were the positive or negative control, respectively.

<sup>d</sup>  $\leq 10$  colonies on plates indicates accumulation of unencapsulated suppressor mutants (see text).

## NptA is the Third P<sub>i</sub> Uptake System That Functions in $\Delta cps \Delta pst1 \Delta pst2$ Mutants

Normal growth of the  $\Delta cps \Delta pst1 \Delta pst2$  mutant in high P<sub>i</sub> medium (Figure 5) implies that sufficient P<sub>i</sub> is being taken up by a third uptake system. BLAST searches did not reveal a close pneumococcal homolog of the Pit symporters of *E. coli* and *B. subtilis*. During these searches, we found another candidate gene, *spd\_0443*, which encodes a putative Na<sup>+</sup>/P<sub>i</sub>-cotransporter II-like protein. Spd\_0443 homologs have been shown to act as P<sub>i</sub> transporters in mammalian intestines and kidneys (Katai et al., 1999) and in certain bacterial species, such as *V. cholerae* and *Vibrio vulnificus* (Lebens et al., 2002; Staley and Harwood, 2014). Consistent with a role in P<sub>i</sub> uptake, we could not delete *spd\_0443* in a  $\Delta cps \Delta pst1 \Delta pst2$  mutant in high P<sub>i</sub> medium (Table 3), but we could delete *spd\_0443* in the  $\Delta pst1$  or  $\Delta pst2$  single mutant (Table 2). Thus, the Spd\_0443 Na<sup>+</sup>/P<sub>i</sub>-cotransporter likely acts as a third P<sub>i</sub> uptake system in *S. pneumoniae*. Because the iron transporter in *S. pneumoniae* is already named “Pit,” we renamed Spd\_0443 as NptA (Na<sup>+</sup>-dependent phosphate transporter A), similar to *Vibrio* species (Lebens et al., 2002).

**TABLE 3 | NptA (Na<sup>+</sup>/P<sub>i</sub> co-transporter) is a third P<sub>i</sub> uptake system.**

| Recipient strain <sup>a</sup> | Amplicon <sup>b</sup>   | Number of colonies on transformation plates after $\approx 20$ h |
|-------------------------------|-------------------------|--|
| $\Delta pst1$ cps             | $\Delta pnpR$ (control) | 150–200 ( $n = 3$ )  |
|                               | $\Delta nptA$           | 100–150 ( $n = 3$ )  |
|                               | No DNA (control)        | 0 ( $n = 3$ )  |
| $\Delta pst2$ cps             | $\Delta pnpR$ (control) | 150–200 ( $n = 3$ )  |
|                               | $\Delta nptA$           | 100–150 ( $n = 3$ )  |
|                               | No DNA (control)        | 0 ( $n = 3$ )  |
| $\Delta pst1 \Delta pst2$ cps | $\Delta pnpR$ (control) | 150–200 ( $n = 3$ )  |
|                               | $\Delta nptA$           | 0 ( $n = 3$ )  |
|                               | No DNA (control)        | 0 ( $n = 3$ )  |

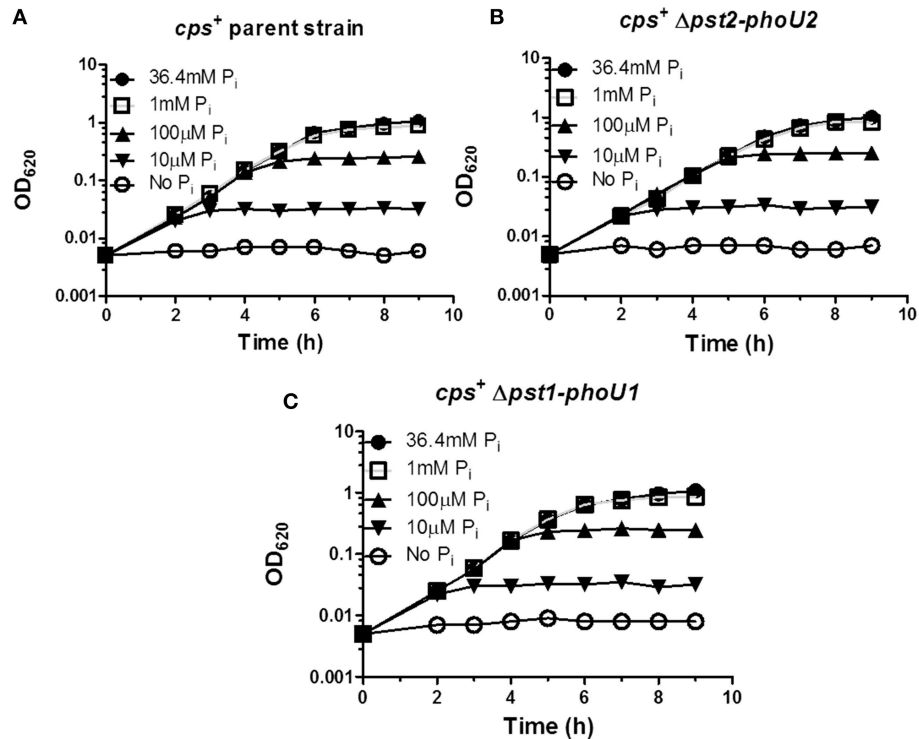
<sup>a</sup> Transformations were performed as described in Materials and Methods. Transformations were performed into two cps mutants [IU1824 (D39  $\Delta cps$  rpsL1); IU3309 (D39  $\Delta cps2E$  rpsL1)] with similar results.

<sup>b</sup> Amplicons were synthesized as described in Materials and Methods (see Table S2). Amplicons used for transformations contained the P<sub>c</sub>-kanrpsL<sup>+</sup> or P<sub>c</sub>-erm antibiotic cassette for selection. Transformations with the  $\Delta pnpR$  amplicon or without DNA were the positive and negative control, respectively.

## $\Delta pst1$ -*phoU1* or $\Delta pst2$ -*phoU2* Deletion Has No Effect on Growth Under Low P<sub>i</sub> Conditions

We next examined the roles of the Pst1 and Pst2 transporters and their regulators (Figure 1) under low P<sub>i</sub> culture conditions. A previous study used the semi-defined C+Y medium as a low P<sub>i</sub> condition (Novak et al., 1999). However, encapsulated strains grew in C+Y broth without P<sub>i</sub> addition (Figure S6), and direct chemical assay (see Materials and Methods; Katewa and Katyare, 2003) showed that C+Y broth (with no added P<sub>i</sub>) contains  $\approx 1.5$  mM P<sub>i</sub> (Figure S7). Therefore, a modified chemically defined medium (mCDM) was used to study low P<sub>i</sub> culture conditions (see Materials and Methods; Carvalho et al., 2013). The growth yield of the wild-type encapsulated strain was dependent on P<sub>i</sub> concentration below 1 mM P<sub>i</sub>, with growth detectable down to  $\approx 10$   $\mu$ M and no growth without P<sub>i</sub> addition (Figure 6A). Growth rates and yields of the  $\Delta pst2$ -*phoU2* and  $\Delta pst1$ -*phoU1* mutants were similar to those of the parent strain (Figures 6B,C), implying that a functional Pst1 or Pst2 transporter is sufficient for optimal growth under low P<sub>i</sub> conditions.

We used unencapsulated ( $\Delta cps$ ) mutants to determine the effects of P<sub>i</sub> concentration when both the Pst1 and Pst2 transporters were absent. Similar to the encapsulated strains (Figure 6), growth yield of the parent,  $\Delta pst1$ -*phoU1*, and  $\Delta pst2$ -*phoU2* single mutants decreased below 1 mM P<sub>i</sub> and was still detectable at 10  $\mu$ M P<sub>i</sub> (Figures 7A–C). By contrast, the growth of the  $\Delta pst2$ -*phoU2*  $\Delta pst1$ -*phoU1* double mutant was not fully supported even by 2 mM P<sub>i</sub>, and growth yield showed apparent autolysis in 1 mM P<sub>i</sub> and no growth with  $\geq 100$   $\mu$ M P<sub>i</sub> (Figure 7D). We conclude that in low P<sub>i</sub> conditions, the NptA transporter alone is not sufficient for growth of D39-derived *S. pneumoniae*, consistent with NptA acting as a low-affinity P<sub>i</sub> transporter compared to Pst1 or Pst2.



**FIGURE 6 |** Deletion of the *pst1-phoU1* or *pst2-phoU2* operon does not affect growth under low P<sub>i</sub> conditions in mCDM media. **(A)** Representative growth curves of encapsulated parent strain (IU1781) in mCDM with 36.4, 1 mM, 100, 10 μM, and no P<sub>i</sub>. **(B)** Representative growth curves of a  $\Delta$ *pst2-phoU2* mutant (IU6550) in mCDM with 36.4, 1 mM, 100, 10 μM, and no P<sub>i</sub>. **(C)** Representative growth curves of a  $\Delta$ *pst1-phoU1* mutant (IU6830) in mCDM with 36.4, 1 mM, 100, 10 μM, and no P<sub>i</sub>. mCDM with different concentrations of P<sub>i</sub> was prepared as described in Materials and Methods. Growth curves were determined at least 3X independently.

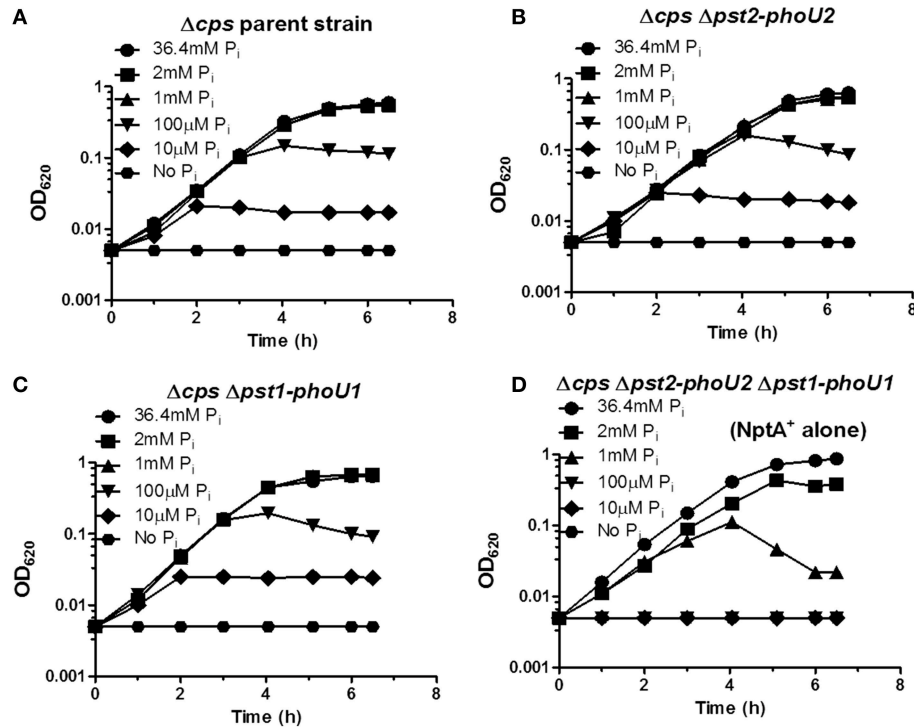
## Low P<sub>i</sub> Induces *pst1* Operon Transcript Amount >100X via Phosphorylation of the PnpR~P RR

We determined the effect of low P<sub>i</sub> concentration on *pst1* operon expression in the encapsulated D39 strain. Strains were grown in high-P<sub>i</sub> (36.4 mM) mCDM, washed, and resuspended in mCDM containing high (36.4 mM) or low (10 μM) P<sub>i</sub> (see Materials and Methods). After 30 min, samples were taken for qRT-PCR using 16S rRNA as the normalization standard (see Materials and Methods). In all strains tested, P<sub>i</sub> limitation to 10 μM induced relative *pst1* transcript amount by >100X compared to the wild-type parent strain in high (36.4 mM) P<sub>i</sub> (Table 4). Similar to strains grown in BHI (Table 1),  $\Delta$ *phoU2* and  $\Delta$ *phoU2*  $\Delta$ *phoU1* mutants showed  $\approx$ 25X induction of *pst1* operon transcript amount when grown in mCDM containing high P<sub>i</sub> (Table 4), whereas a  $\Delta$ *phoU1* mutant showed no increase (Table 4). In these mutants, reduction of P<sub>i</sub> concentration to 10 μM induced *pst1* transcript amounts by an additional  $\approx$ 5–100X (Table 4). P<sub>i</sub> limitation of a  $\Delta$ *pnpRS* TCS mutant showed no increase in relative *pst1* operon transcript amounts (data not shown), indicating a dependence on the PnpRS TCS for induction of *pst1* operon transcription in low P<sub>i</sub> media. This conclusion was confirmed by Phos-tag SDS PAGE, which showed that phosphorylation of the PnpR RR (PnpR~P) went from <1% in mCDM containing 36.4 mM P<sub>i</sub> to  $\approx$ 80% following a shift

to mCDM lacking P<sub>i</sub> for 40 min (Figure S8A). We conclude that transcription of pneumococcal *pst1* transporter operon is strongly induced by the PnpR~P RR in low P<sub>i</sub> conditions.

## Deletion of *pst1-phoU1* and *pst2-phoU2* Reduces the Rate of P<sub>i</sub> Uptake by $\approx$ 50% in an Unencapsulated Strain

Previously, the relative rate of P<sub>i</sub> uptake was reported to be reduced by  $\approx$ 30% in a  $\Delta$ *pstB1* mutant of laboratory strain R6x grown in C+Y medium (no added P<sub>i</sub>) (Novak et al., 1999), which turns out to contain a relatively high (1.5 mM) P<sub>i</sub> content (above; Figure S7). To the contrary, <sup>32</sup>P<sub>i</sub> uptake experiments in encapsulated and unencapsulated D39 mutants lacking the Pst1 ( $\Delta$ *pst1-phoU1*) or Pst2 ( $\Delta$ *pst2-phoU2*) transporter showed linear rates of <sup>32</sup>P<sub>i</sub> uptake for at least 10 min that were similar to those of the wild-type parent strains in mCDM containing 1 mM total P<sub>i</sub> (Figures 8A,B, Table S7). R6x is a derivative of R6 (Tiraby and Fox, 1973), which is an unencapsulated derivative of D39 (Lanie et al., 2007). The difference between this and the previous result (Novak et al., 1999) may partly reflect the large number of mutations in the R6 laboratory strain compared to the D39 strain progenitor genetic background (Lanie et al., 2007). In contrast to the single mutants, the unencapsulated  $\Delta$ *pst1-phoU1*  $\Delta$ *pst2-phoU2* double mutant showed a significant drop ( $\approx$ 50%) in the rate of P<sub>i</sub> uptake compared to the wild-type parent strain or



**FIGURE 7 | The NptA  $\text{Na}^+/\text{P}_i$  co-transporter alone cannot provide sufficient  $\text{P}_i$  for long-term growth in mCDM containing moderately high (1 mM) and lower  $\text{P}_i$  concentrations. (A)** Representative growth curves of unencapsulated parent strain  $\Delta\text{cps}$  (IU1945) in mCDM with 36.4, 2, 1 mM, 100, 10  $\mu\text{M}$ , and no  $\text{P}_i$ . **(B)** Representative growth curves of unencapsulated  $\Delta\text{cps}$   $\Delta\text{pst2-phoU2}$  mutant (K583) in mCDM with 36.4, 2, 1 mM, 100, 10  $\mu\text{M}$ , and no  $\text{P}_i$ . **(C)** Representative growth curves of unencapsulated  $\Delta\text{cps}$   $\Delta\text{pst1-phoU1}$  mutant (E595) in mCDM with 36.4, 2, 1 mM, 100, 10  $\mu\text{M}$ , and no  $\text{P}_i$ . **(D)** Representative growth curves of unencapsulated  $\Delta\text{cps}$   $\Delta\text{pst2-phoU2}$   $\Delta\text{pst1-phoU1}$  mutant (IU5774), which only contains the NptA  $\text{Na}^+/\text{P}_i$  transporter, in mCDM with 36.4, 2, 1 mM, 100, 10  $\mu\text{M}$ , and no  $\text{P}_i$ . Growth curves were determined at least 3X independently.

the single mutants in mCDM containing 1 mM  $\text{P}_i$  (Figure 8B, Table S7) or in C+Y broth (data not shown). This result implicates both the Pst1 and Pst2 transporters in  $\text{P}_i$  uptake in *S. pneumoniae* D39.

### $\text{P}_i$ Uptake is Reduced in a $\Delta\text{pst1-phoU1}$ Mutant Limited for Total $\text{P}_i$

To determine  $\text{P}_i$  uptake under low  $\text{P}_i$  conditions, we incubated encapsulated parent,  $\Delta\text{pst1-phoU1}$ , or  $\Delta\text{pst2-phoU2}$  mutants in mCDM lacking  $\text{P}_i$  for 1 h, and then added  $^{32}\text{P}_i$  (total  $[\text{P}_i] = 200 \mu\text{M}$ ), and sampled  $\text{P}_i$  uptake with time (Figure 8C). Unlike the constant rate of  $^{32}\text{P}$  uptake observed at 1 mM total  $\text{P}_i$  (Figures 8A,B),  $\text{P}_i$  uptake leveled off after about 5 min in 200  $\mu\text{M}$   $\text{P}_i$  (Figure 8C). The initial rate of  $\text{P}_i$  uptake by the encapsulated parent strain was markedly greater ( $\approx 8\text{X}$ ) in 200  $\mu\text{M}$   $\text{P}_i$  than in 1 mM  $\text{P}_i$  (Figures 8A,C, Tables S7, S8). The initial rate of  $\text{P}_i$  uptake was comparable for the parent strain and the  $\Delta\text{pst2-phoU2}$  mutant (Figure 8C, Table S8). In contrast, the initial rate of  $\text{P}_i$  uptake was reduced by  $\approx 2\text{X}$  in the  $\Delta\text{pst1-phoU1}$  mutant compared to the parent or  $\Delta\text{pst2-phoU2}$  mutant (Figure 8C, Table S8). These results support a primary role for the Pst1 transporter in low- $\text{P}_i$  conditions.

## DISCUSSION

In this paper we demonstrate for the first time that regulation of the two evolutionarily distinct Pst  $\text{P}_i$  transport systems is linked in several ways in the human commensal and pathogen, *S. pneumoniae*. Niches encountered by pneumococcus in human hosts contain different  $\text{P}_i$  concentrations, including nasal fluid ( $\approx 5 \text{ mM}$ ), saliva ( $\approx 1 \text{ mM}$ ), and serum ( $\approx 1 \text{ mM}$ ) (Bansal, 1990; Wilson, 2005, 2008). Several aspects of this regulatory network are unusual, compared to the usual negative regulation of  $\text{P}_i$  uptake even at moderate  $\text{P}_i$  concentrations in other bacterial species (Figure 1; Hulett, 1993; Hsieh and Wanner, 2010; Botella et al., 2011). The *pst1-phoU1* and *pst2-phoU2* operons are completely separated in the pneumococcal chromosome, and although upstream of the *pst1-phoU1* operon, the *pnpRS* operon, which encodes the PnpRS TCS, is independently expressed and not autoregulated (Table 1, Figure 1). Deletion of gene *spd\_1614*, which encodes a third putative PhoU-like protein, did not lead to growth phenotypes and was not studied further (data not shown).

Expression of the Pst2 transporter is constitutive under the conditions tested here, including media containing high  $\text{P}_i$  concentrations (Table 1, Table S6, and Figure S3). However, Pst2 uptake of  $\text{P}_i$  is likely negatively regulated by the PhoU2 protein, whose absence causes a drop in growth yield and



**TABLE 4 | Relative transcript amounts of the *pst1* operon in different mutants in mCDM containing high and low  $P_i$  concentrations<sup>a</sup>.**

| Strains <sup>b</sup>                                 | $P_i$ concentration in mCDM | Relative transcript amount of <i>pstS1</i> operon <sup>c</sup> |
|--|-----------------------------|--|
| Parent strain (IU1781)                               | 36.4 mM                     | ≡1   |
|  | 10 $\mu$ M                  | 209.7 $\pm$ 37.9 (n = 6) (***) <sup>d</sup>                    |
| $\Delta$ <i>phoU2</i> (IU6375)                       | 36.4 mM                     | 24.1 $\pm$ 3.9 (n = 3) (***)                                   |
|  | 10 $\mu$ M                  | 201.6 $\pm$ 47.6 (n = 3) (***)                                 |
| $\Delta$ <i>phoU1</i> (IU6377)                       | 36.4 mM                     | 1.4 $\pm$ 0.1 (n = 3) (ns)                                     |
|  | 10 $\mu$ M                  | 145.4 $\pm$ 25.3 (n = 4) (***)                                 |
| $\Delta$ <i>phoU2</i> $\Delta$ <i>phoU1</i> (IU6499) | 36.4 mM                     | 28.3 $\pm$ 0.5 (n = 3) (***)                                   |
|  | 10 $\mu$ M                  | 136.3 $\pm$ 12.6 (n = 3) (***)                                 |

<sup>a</sup>RNA preparation and qRT-PCR from strains in mCDM containing high and low  $P_i$  concentrations were performed as described in Materials and Methods.

<sup>b</sup>Strains were markerless deletion mutants derived from encapsulated parent strain IU1781 (see Table S1).

<sup>c</sup>Relative *pstS1* gene transcript amount was used to represent *pst1* operon expression.

<sup>d</sup>\*\*\* $P < 0.001$ ; ns, not significant.  $P$ -values were calculated by an unpaired  $t$ -test in GraphPad Prism.

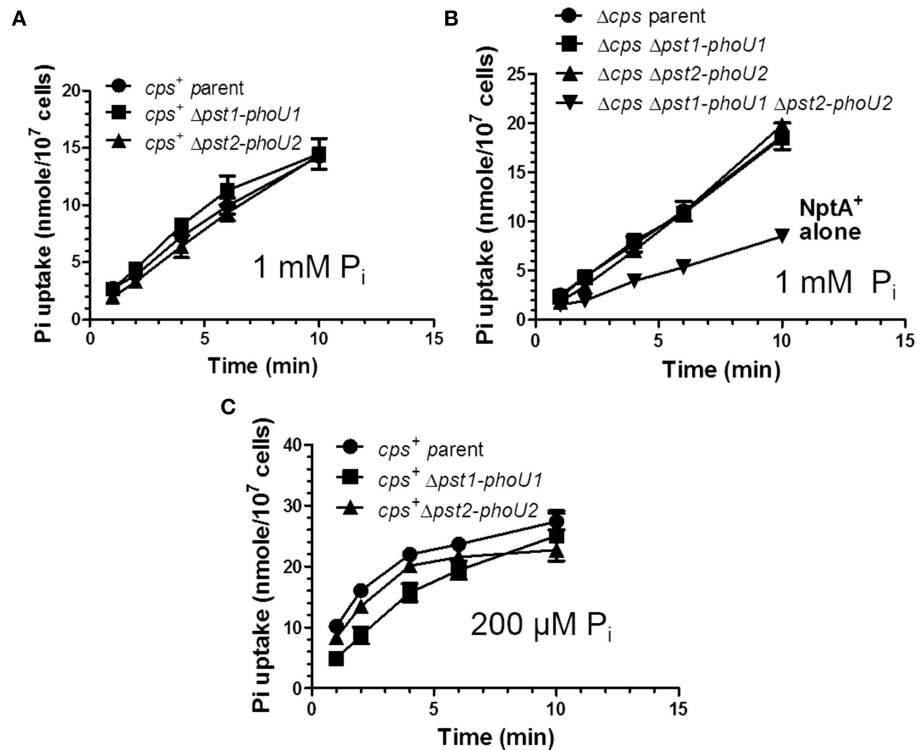
increased antibiotic sensitivity in mutants growing in high  $P_i$  medium (Tables S4, S5, Figures 1, 2, and Figure S3). In addition, PhoU2 negatively regulates the transcription activation of the *pst1-phoU1* operon by the phosphorylated PnpR~P RR, such that a  $\Delta$ *phoU2* deletion mutation leads to a substantial increase in *pst1-phoU1* transcript amounts (Table 1, Figures 1, 3, and Figure S4). The PhoU1 protein, which shares only 34% amino acid identities with PhoU2, does not play a reciprocal role in negatively regulating *pst1-phoU1* transcription (Table 1, lines 3, 11, and 14), but PhoU1 likely negatively regulates  $P_i$  uptake by the Pst1, but not the Pst2,  $P_i$  transporter (Figures 1, 4, 5). Thus, the PhoU2 protein can regulate both PnpR phosphorylation level and Pst2 transporter function, similar to the PhoU homolog in *E. coli* (Hsieh and Wanner, 2010; Gardner et al., 2014, 2015). In contrast, PhoU1 function is restricted to regulating transporter but not TCS function, similar to the PhoU in *C. crescentus* (Lubin et al., 2016). *E. coli* PhoU interacts with the PAS domain of the PhoR HK (Gardner et al., 2014, 2015). However, the pneumococcal PnpS HK lacks a recognizable PAS domain, and it remains to be determined whether PhoU2 regulation of PnpR~P levels is through direct interactions with the PnpS HK. Likewise, it is unknown whether PhoU2 interacts with the PstB1 subunit of the Pst1 transporter to exert control over *pst1-phoU1* operon transcription, by analogy to control in *E. coli* (Gardner et al., 2014, 2015). Interactions of PhoU1 or PhoU2 with subunits of the Pst1 or Pst2 transporters also remain to be determined.

The requirement of a functional Pst1 or Pst2 for growth of the encapsulated serotype 2 strain provides a biological rationale for the constitutive expression and function of the Pst2 transporter at high  $P_i$  concentrations (Table 2) and why *S. pneumoniae* maintains dual  $P_i$  uptake systems. Capsule is one of the most important factors required for pneumococcal colonization, carriage, and virulence in its human host (Briles et al., 1992;

Morona et al., 2004, 2006; Bentley et al., 2006; Hyams et al., 2010). Expression of the Pst1 transporter is strongly induced by low  $P_i$  conditions (Table 4), and lack of PhoU1 does not change *pst1-phoU1* operon expression in high  $P_i$  media (Tables 2, 4). These results, and a reduced rate of  $P_i$  uptake by mutants lacking Pst1 (Figure 8C, Table S8) indicate that Pst1 functions mainly at low  $P_i$  concentrations. However, encapsulated *S. pneumoniae* strains require high-affinity  $P_i$  transport even in high- $P_i$  media (Table 2), and this  $P_i$  uptake cannot be provided by the low-affinity NptA  $Na^+/P_i$  cotransporter that replaces Pst1 and Pst2 in unencapsulated strains (Figure 8B, Table S7). The reason underlying this link between Pst-mediated  $P_i$  transport and the maintenance of capsule is not currently clear. A recent report suggests that low  $P_i$  conditions induce capsule biosynthesis in *M. tuberculosis* (van de Weerd et al., 2016). Overexpression of capsule could alter the metabolism of *S. pneumoniae* thereby inhibiting the growth of *cps+*  $\Delta$ *pst1*  $\Delta$ *pst2* mutants and leading to the appearance of spontaneous *cps* mutants (Table 2). However, starvation of the wild-type D39 *cps+* strain for  $P_i$  for 1 h did not reveal a qualitative change in capsule amount in the Quellung reaction (data not shown). Moreover, pneumococcal capsule biosynthesis is positively regulated by phosphorylation of regulatory protein CpsD (Yother, 2011), and this protein phosphorylation would likely be reduced during  $P_i$  limitation.

The regulatory pathways that mediate capsule induction in *M. tuberculosis* involve sigma factors and poly- $P_i$  kinases (van de Weerd et al., 2016) that are absent from *S. pneumoniae*. As mentioned in the Introduction,  $\Delta$ *phoU* mutants of other bacteria accumulate poly- $P_i$  (Morohoshi et al., 2002; Hirota et al., 2013; Wang et al., 2013; de Almeida et al., 2015; Lubin et al., 2016), possibly because high intracellular  $P_i$  concentration disrupt metabolic homeostasis leading to defects in growth accompanied by general antibiotic sensitivity. RNA-Seq results reported here indicate that the pneumococcal Pho regulon contains a limited number of recognizable genes involved in  $P_i$  accumulation (Table S6). Notably, *S. pneumoniae* serotype 2 strain D39 as well as some other serotype strains, such as TIGR4, lack recognizable homologs of poly- $P_i$  kinases Ppk1 and Ppk2 (Zhang et al., 2002) and  $Mg^{2+}$ -dependent poly- $P_i$  exopolyphosphatase Ppx (Akiyama et al., 1993), but do encode a degradative  $Mn^{2+}$ -dependent, inorganic pyrophosphatase (PpaC) (Lanie et al., 2007); therefore, it is unclear whether these *S. pneumoniae* strains produce poly- $P_i$ . DAPI staining experiments that revealed poly- $P_i$  accumulation in *C. crescentus*  $\Delta$ *phoU* mutants (Lubin et al., 2016) were inconclusive and did not indicate any differences in staining of the *phoU2*<sup>+</sup> parent and  $\Delta$ *phoU2* mutant in high- $P_i$  BHI broth (data not shown). Similarly, DAPI-based assays of extracts did not show any difference between the parent and  $\Delta$ *phoU2* mutant indicative of different poly- $P_i$  amounts (data not shown).

Taken together, our results suggest that *S. pneumoniae* may maintain the regulated Pst1 and constitutive Pst2  $P_i$  transport systems as a failsafe to ensure capsule biosynthesis is maintained during variations in  $P_i$  conditions. Coordination between the two Pst  $P_i$  transport systems is coordinated by the PhoU2 protein that modulates transcription of the *pst1-phoU1* operon by the PnpRS TCS and separately regulates the function of



**FIGURE 8 | The Pst1 or Pst2 transporter is sufficient for P<sub>i</sub> uptake under moderately high P<sub>i</sub> conditions (1 mM), but the Pst1 transporter is required for optimal P<sub>i</sub> uptake under low P<sub>i</sub> conditions (200  $\mu$ M) following P<sub>i</sub> limitation. (A) P<sub>i</sub> uptake assays of encapsulated parent strain (IU1781) and  $\Delta$ *pst2-phoU2* (IU6550) and  $\Delta$ *pst1-phoU1* (IU6830) mutants in 1 mM total <sup>32</sup>P<sub>i</sub> in mCDM (*n* = 3). P<sub>i</sub> uptake was determined as the initial rate of <sup>32</sup>P<sub>i</sub> incorporation into cells in mCDM in pulse-labeling experiments described in Materials and Methods. See Tables S7 for initial rates of P<sub>i</sub> uptake (*n* = 3). (B) P<sub>i</sub> uptake assays of unencapsulated parent strain  $\Delta$ *cps* (IU1945) and  $\Delta$ *cps*  $\Delta$ *pst2-phoU2* (K583),  $\Delta$ *cps*  $\Delta$ *pst1-phoU1* (E595), and  $\Delta$ *cps*  $\Delta$ *pst1-phoU1*  $\Delta$ *pst2-phoU2* (IU5774) mutants in 1 mM P<sub>i</sub> mCDM. See Tables S7 for initial rates of P<sub>i</sub> uptake (*n* = 3). (C) P<sub>i</sub> uptake assay of encapsulated parent strain (IU1781) and  $\Delta$ *pst2-phoU2* (IU6550) and  $\Delta$ *pst1-phoU1* (IU6830) mutants in 200  $\mu$ M total P<sub>i</sub> mCDM following P<sub>i</sub> starvation for 1 h as described in Materials and Methods (*n* = 3). Initial rates of P<sub>i</sub> uptake were determined with the first min of labeling and are listed in Table S8 (*n* = 3).**

the Pst2 transporter. Most bacteria encode a single Pst-type P<sub>i</sub> transporter as part of a Pho regulon that encode numerous phosphate accumulation and sparing proteins (Hulett, 1993; Wanner, 1996; Hsieh and Wanner, 2010). The limited Pho regulon of *S. pneumoniae* may reflect restriction to the human host, where P<sub>i</sub> is the primary source of phosphorus (Wilson, 2008). Even among the *Streptococcus*, dual Pst systems are limited to only a few species, including *S. pneumoniae*, *S. pseudopneumoniae*, *S. dysgalactiae*, *S. equi* (Group C), *S. porcinus*, *S. agalactiae* (Group B), and *S. equinus*, and are absent from the major Viridans and A Groups. Outside of the *Streptococcus*, multiple Pst P<sub>i</sub> transporters have only been reported in a very limited number of bacteria, including *Synechocystis* sp. PCC 6803 and *M. tuberculosis* (Braibant et al., 1996; Suzuki et al., 2004; Burut-Archanai et al., 2011; Tischler et al., 2013). In *Synechocystis*, low P<sub>i</sub> conditions activate expression of both of the Pst P<sub>i</sub> transporters that are present (Suzuki et al., 2004), whereas in *M. tuberculosis* one Pst transporter is activated by low P<sub>i</sub> and at least one other Pst transporter seems to be constitutively expressed, like Pst2 in *S. pneumoniae* (Tischler et al., 2013).

## AUTHOR CONTRIBUTIONS

JZ, DS, KW, MW contributed to the conception and design of the work; JZ, DS carried out acquisition and analysis of data; JZ, DS, KW, MW interpreted data for the work; JZ, MW drafted and wrote the final version of the paper; JZ, DS, KW, MW approved the final version to be published.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fcimb.2016.00063>

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