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GbdR Regulates *Pseudomonas aeruginosa plcH* and *pchP* Transcription in Response to Choline Catabolites▽†

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Pseudomonas aeruginosa **hemolytic phospholipase C, PlcH, can degrade phosphatidylcholine (PC) and sphingomyelin in eukaryotic cell membranes and extracellular PC in lung surfactant. Numerous studies implicate PlcH in *P. aeruginosa* virulence. The phosphorylcholine released by PlcH activity on phospholipids is hydrolyzed by a periplasmic phosphorylcholine phosphatase, PchP. Both *plcH* gene expression and PchP enzyme activity are positively regulated by phosphorylcholine degradation products, including glycine betaine. Here we report that the induction of *plcH* and *pchP* transcription by glycine betaine is mediated by GbdR, an AraC family transcription factor. Mutants that lack *gbdR* are unable to induce *plcH* and *pchP* in media containing glycine betaine or choline and in phosphatidylcholine-rich environments, such as lung surfactant or mouse lung lavage fluid. In T broth containing choline, the *gbdR* mutant exhibited a 95% reduction in PlcH activity. In electrophoretic mobility shift assays, a GbdR-maltose binding protein fusion bound specifically to both the *plcH* and *pchP* promoters. Promoter mapping, alignment of GbdR-regulated promoter sequences, and analysis of targeted promoter mutants that lack GbdR-dependent induction of transcription were used to identify a region necessary for GbdR-dependent transcriptional activation. GbdR also plays a significant role in *plcH* and *pchP* regulation within the mouse lung. Our studies suggest that GbdR is the primary regulator of *plcH* and *pchP* expression in PC-rich environments, such as the lung, and that *pchP* and other genes involved in phosphorylcholine catabolism are necessary to stimulate the GbdR-mediated positive feedback induction of *plcH*.**

The *Pseudomonas aeruginosa plcH* gene encodes a hemolytic phospholipase C (PLC) with high specificity for phosphatidylcholine (PC) and sphingomyelin (SM) (4). Studies have shown PlcH to have both pathogenic activities and proinflammatory capability (3, 21, 45). In addition, PlcH is important for *P. aeruginosa* virulence in mice (29), rabbits (46), insects (12), plants (28, 29), and *Candida albicans* (10). Purified PlcH is capable of inducing pulmonary inflammation and inhibits the oxidative burst of neutrophils (40, 45). Evidence also suggests that *P. aeruginosa* PlcH-mediated degradation of the PC in lung surfactant contributes to lung infections (16).

The enzymatic activity of PlcH on either PC or SM releases phosphorylcholine (ChoP), which can be dephosphorylated in the periplasm by a phosphatase, PchP, to yield choline (20). Choline is actively transported into the cytoplasm (19, 33), where it can be used in a variety of different ways. *P. aeruginosa* BetAB catalyzes the conversion of choline to glycine betaine (GB), which is both a potent osmoprotectant (9) and a source of carbon, nitrogen, and energy (13). During catabolism of GB, it is successively demethylated to form dimethylglycine (DMG), sarcosine (monomethylglycine), and finally glycine (7, 43). Microarray studies of cystic fibrosis (CF) patient sputum samples showed that in the lungs of CF patients, *P. aeruginosa* induces *plcH* and genes involved in both choline and diacylglycerol catabolism, which supports the hypothesis that PlcH is important for *P. aeruginosa* colonization of the lung (39).

PlcH activity provides the *P. aeruginosa* cell with ChoP, a source of both phosphate and choline, and both of these products participate in the regulation of *plcH* transcription (36). Shortridge et al. (36) have shown that *plcH* transcription is induced by phosphate limitation in a PhoB-dependent manner. The *plcH* gene is also transcriptionally induced in response to choline and its downstream catabolic products, GB and DMG (32, 36). Induction of *plcH* by GB and DMG is PhoB independent, and there is evidence for distinct transcriptional start sites for phosphate limitation-induced and choline-induced transcripts (32, 36, 41). The inability of choline to induce *plcH* in a mutant defective for *betB*, required for conversion of choline to GB, provided evidence that choline is not a direct activator of *plcH* transcription, but rather that GB and DMG are the inducers (31). The transcription factor responsible for this induction has not previously been described.

Here we show that GbdR, an AraC family transcription factor that is required for the induction of the GB and DMG catabolic genes in response to GB and DMG (43), also regulates the expression of *plcH* and *pchP* in a similar manner. Through deletion mapping, electrophoretic mobility shift assays, and targeted promoter mutagenesis, we have identified residues that are required for GB- and DMG-dependent induction of *plcH* and *pchP*. We show that GbdR is critical for

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TABLE 1. Strain and plasmid list

Strain or plasmid	Strain no.	Description
Strains		
P. aeruginosa strains		
PAO1	DH20	*P. aeruginosa* WT
Δ*gbdR*	DH543	In-frame PA5380 deletion in PAO1 (43)
Δ*gbdR att*::*gbdR*	DH1008	Complementation of the *gbdR* deletion at the *att* site
Δ*gbcAB*	DH841	In-frame PA5410 and PA5411 deletions in PAO1 (43)
Δ*dgcA*	DH1178	In-frame PA5398 deletion in PAO1 (43)
Δ*plcHR*	DH860	In-frame *plcHR* deletion in PAO1 (36)
pchP::Tn*5*	DH503	PAO1 transposon mutant 10802
betB::Tn*5*	DH491	PAO1 transposon mutant 104
PA14	DH122	*P. aeruginosa* WT (31)
Δ*gbdR*	DH466	In-frame PA5380 deletion in PA14 (43)
E. coli strains		
S17/Δ*pir*	DH522	
Ec-*PA5380*KO	DH540	DH522 with p*PA5380*KO; Gm^r
Plasmids		
pUCP22		High-copy-number *Pseudomonas* stabilized vector; Gm^r (34)
pMQ30		Suicide vector; Gm^r (35)
pMQ80		High-copy-number *Pseudomonas*-yeast shuttle vector (35)
pMW5		pUCP22 containing *lacZYA* from pRS415
pMW22		*plcH* promoter fragment (positions −374 to −13) in pMW5
pMW23		*plcH* promoter fragment (positions −248 to −13) in pMW5
pMW24		*plcH* promoter fragment (positions −177 to −13) in pMW5
pMW25		*plcH* promoter fragment (positions −86 to −13) in pMW5
pMW26		MBP-GbdR expression vector (pMalc derivative)
pMW47		GFP reporter vector based on modified pMQ80
pMW71		*pchP* promoter fragment (positions −369 to −15) in pMW47
pMW72		*pchP* promoter fragment (positions −275 to −15) in pMW47
pMW73		*pchP* promoter fragment (positions −141 to −15) in pMW47
pMW84		*pchP* promoter fragment (positions −75 to −15) in pMW47

induction of *plcH* and *pchP* in response to bovine surfactant and mouse bronchoalveolar lavage fluid (BALF). Analysis of *P. aeruginosa* transcripts during acute murine lung infection also indicated that GbdR is important for the induction of *plcH* and *pchP* in vivo.

MATERIALS AND METHODS

Strains, media, and growth conditions. *P. aeruginosa* PAO1 and PA14 wild-type (WT) strains and deletion strains, as well as *Escherichia coli* strains (Table 1), were maintained on Luria broth (LB) medium. For transcriptional induction studies, cells were grown overnight in morpholinepropanesulfonic acid (MOPS) minimal medium (24) amended with 25 mM sodium pyruvate and 5 mM D-glucose prior to transfer to inducing medium. When necessary, gentamicin was added to a final concentration of 10 μg/ml for *E. coli*, 50 μg/ml for *P. aeruginosa* in LB medium, and 25 μg/ml for *P. aeruginosa* in MOPS medium.

PLC activity assays. PLC activity was measured using the artificial substrate ρ-nitrophenyl-phosphorylcholine (NPPC) by the method described by Kurioka and Matsuda (15). Bacteria were grown overnight in 5 ml of tryptone broth or tryptone broth plus 5 mM choline overnight at 37°C. The reaction buffer was 100 mM Tris-HCl (pH 7.2), 25% glycerol, and 20 mM NPPC. NPPC hydrolysis was detected by measuring the absorbance at 410 nm. Hemolytic activity was analyzed on agar plates containing 5% defibrinated sheep blood. To eliminate *plcH* induction in response to phosphate limitation, 500 μl of 1 M potassium phosphate (pH 7.0) solution was top spread and the plates were allowed to dry prior to inoculation with *P. aeruginosa* strains. After 24 h of incubation at 37°C, hemolytic activity was detected by clear halos, which were photographed after removal of the bacteria from the plate by scraping with a coverslip.

RNA isolation, RT, and qRT-PCR. For in vitro gene induction experiments using reverse transcription-PCR (RT-PCR), cells were grown overnight in MOPS with 20 mM pyruvate and 5 mM glucose. Cells were harvested by centrifugation, resuspended in MOPS with 20 mM pyruvate and 10 mM of the inducing carbon source, and grown for 2 h at 37°C. RNAs were isolated from ~10^7 cells by use of an RNeasy kit (Qiagen). Pyruvate served as a growth substrate to allow growth without detectable catabolite repression of GB-induced genes (7; data not shown). During isolation, RNAs were treated with an on-column DNase I treatment (Qiagen) for 30 min at room temperature. The resulting RNAs were subjected to PCR to verify the absence of contaminating DNA before being quantified using a Nanodrop spectrophotometer. cDNA was synthesized using Superscript III (Invitrogen) from 300 ng of starting RNA, with a 5′-NSNSNSNSNS-3′ primer instead of random hexamers. The regimen for cDNA synthesis was 25°C for 5 min, 52°C for 60 min, and 70°C for 15 min. Primers used are listed in Table S1 in the supplemental material. The PCR regimen was 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 30 s. Quantitative real-time RT-PCR (qRT-PCR) was conducted with Power SYBR green with AmpliTaq Gold DNA polymerase according to the manufacturer's instructions (Applied Biosystems).

Analysis of *plcH* promoter activities by use of *lacZYA* fusions. The pMW5 reporter construct was generated by amplifying the *lacZYA* operon from plasmid pRS415 (37), using specific primers with KpnI and EcoRI sites, and digesting the product with KpnI and EcoRI, followed by ligation into similarly digested pUCP22 (34). This placed the *lacZYA* operon in an orientation opposite that of the pUCP22 *lacZ* alpha fragment and the corresponding promoter, leaving the majority of the polycloning region intact. This construct shows very little background β-galactosidase activity in *P. aeruginosa*.

We constructed a deletion series of the *plcH* promoter by using PCR to amplify products from the pAES110 *plcH*::*lacZYA* fusion construct (32). Primers used for these constructs are detailed in Table S1 in the supplemental material. Briefly, each PCR product was gel purified, digested with XbaI and BamHI, and ligated into similarly digested pMW5. Ligation mixtures were transformed into *E. coli* DH5α cells (Invitrogen) by chemical transformation. Plasmid preparations from *E. coli* clones were transformed into *P. aeruginosa* by electroporation (5) and selected for growth on gentamicin. After overnight growth in MOPS-pyruvate-glucose medium as described above, cells were pelleted and resuspended in MOPS with 25 mM pyruvate and 2 mM of the inducing compound and grown under inducing conditions for 4 h at 37°C unless otherwise specified. The β-galactosidase assays were conducted according to the method of Miller (23).

Analysis of *plcH* reporter expression in Survanta (Abbott) was done using the specified *P. aeruginosa* strains carrying the pMW22 reporter plasmid. Survanta was diluted in MOPS with no additional carbon source (1:100). Additions of phosphorylcholine, choline, or GB (all from Sigma) were such that the final concentration of each additive was 0.25 mM. This concentration approximates that which would be derived if all PC in Survanta were hydrolyzed and every subsequent enzymatic step proceeded to completion.

Analysis of the *pchP* promoter by use of promoter fusions to *gfp*. The *pchP* promoter deletion series was constructed by amplifying fragments from PAO1 genomic DNA. Primers for these constructs are detailed in Table S1 in the supplemental material. pMW47 was generated by amplification of the *gfp-mut3* gene from pMQ80 (35) with adaptor primers. This product was digested with HindIII and EcoRI and ligated to a similarly cut pMQ80 backbone. This left the *gfp* gene in reverse orientation to the arabinose-inducible promoter region of pMQ80 and allowed yeast-based cloning of promoters in front of *gfp*. Briefly, each PCR product was gel purified and transformed along with KpnI-linearized pMW47 into *Saccharomyces cerevisiae* according to the method of Shanks et al. (35). Yeast cells were plated onto synthetic complete plates lacking uracil, and colonies were picked after 48 h at 30°C. Plasmid DNA was isolated from yeast according to the method of Shanks et al. (35). Transformation of the plasmids

into *E. coli* and mobilization into *P. aeruginosa* were done as described for the *plcH* promoter fusion constructs. Cells were induced as described for the *plcH* promoter studies. Fluorescence was measured in black-walled 96-well dishes, using a Bio-Tek Instruments model Fix-800I fluorometer with an excitation wavelength of 485 nm and a 528-nm emission filter. Background autofluorescence of PAO1 was subtracted by measuring the fluorescence of identically grown cells carrying a Gm^r plasmid but no *gfp* gene.

EMSA. We constructed a maltose binding protein (MBP) fusion to GbdR (pMW26) by using the pMALc vector system (NEB). *E. coli* DH5α carrying the pMW26 plasmid was grown overnight in LB plus 120 μg/ml carbenicillin. The overnight culture was transferred to four 250-ml flasks containing 50 ml of LB-carbenicillin and shaken at 220 rpm for 4 h. IPTG (isopropyl-β-D-thiogalactopyranoside) was added to 0.2 mM, and the cells were induced for 5 h. Cells were pelleted, lysed in column buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA) with 3 mg/ml lysozyme, clarified by centrifugation, and filtered through a 0.22-μm membrane before being applied to an amylose column. The column was washed with eight column volumes of column buffer, and protein was eluted in column buffer with 10 mM maltose. The fractions containing the fusion protein were dialyzed against 20 mM Tris-HCl, pH 7.5, 125 mM KCl, 1 mM $MgCl_2$, 5% glycerol at 4°C in a dialysis cassette (10,000-molecular-weight cutoff; Pierce). The full-length fusion protein was used in electrophoretic mobility shift assays (EMSAs), as the Xa cleavage product was insoluble (data not shown). We verified the ability of MBP-GbdR to function in vivo by the restoration of growth on GB to the Δ*gbdR* mutant by a plasmid expressing the pMAL-GbdR fusion (data not shown).

DNA fragments for EMSA analysis were created by PCR amplification, verification of the presence of a single band, and spot dialysis of the product. DNA was diluted to the specified concentration from this preparation. For labeled DNA, one of the primers contained a covalently linked 5′-biotin tag (IDT). EMSA experiments were conducted per the manufacturer's instructions (Pierce LightShift), with the following changes. First, buffer conditions were optimized to be similar to those used for other *Pseudomonas* AraC family proteins (22). This resulted in a final binding reaction buffer containing 1× Pierce binding buffer (10 mM Tris-HCl, pH 7.5, 50 mM KCl, 1 mM dithiothreitol), 0.1 mM GB, 0.2 μg/μl poly(dI-dC), 3 mM $MgCl_2$, 7% glycerol, 1 mM EDTA, and 50 μg/ml bovine serum albumin. To reduce the formation of insoluble precipitates with the *pchP* probe, bovine serum albumin was increased to 250 μg/ml. Where included, unlabeled competitor DNA was present at 0.6 pmol/μl, labeled probe DNA was present at 1 fmol/μl, and MBP-GbdR was added to a final concentration of 0 to 3.0 μM. Binding reactions were carried out at 37°C for 40 min. Binding reactions were run in nondenaturing 5% Tris-borate-EDTA gels (Bio-Rad), transferred to BioDyne nylon membranes (Pierce), and detected using the supplied streptavidin-horseradish peroxidase protocol (Pierce).

In vitro *P. aeruginosa* gene induction in response to bovine surfactant and murine BALF. For in vitro surfactant and BALF experiments, *P. aeruginosa* was pregrown in MOPS medium as described for qRT-PCR. For the surfactant study, cells from overnight cultures were pelleted and resuspended in either MOPS plus 10 mM pyruvate or a 1:50 dilution of Survanta in MOPS plus 10 mM pyruvate, resulting in a phospholipid concentration of 0.5 mg/ml total phospholipid. BALF for in vitro *P. aeruginosa* gene induction studies was harvested from uninfected adult male C57BL/6 mice. Mice were anesthetized with intraperitoneal sodium pentobarbital (70 to 90 mg/kg of body weight). Tracheas were cannulated with 2 cm of 22-gauge polyethylene tubing attached to a 23-gauge needle, 1 ml of cold Dulbecco's phosphate-buffered saline (DPBS) was instilled into the lungs, and the BALF was collected. The BALF was sterile filtered and frozen at −80°C until use. For the BALF experiment, cells were resuspended in DPBS plus 10 mM pyruvate or into BALF plus 10 mM pyruvate. For both analyses, cells were shaken at 37°C for 2 h and RNAs were prepared as described above.

Mouse lung infection. Male C57BL/6 mice (Jackson Labs) of 8 to 12 weeks of age were inoculated with 1×10^8 CFU of *P. aeruginosa* PAO1 via oropharyngeal aspiration following brief anesthesia with isoflurane (1). The infection proceeded for 24 hours, followed by nonsurvival surgery and acquisition of BALF by the same methods described above for uninfected animals. Samples were immediately treated with RNAprotect bacterial reagent (Qiagen) per the manufacturer's instructions. RNA isolation was performed as described above, with the following change: 95 to 160 ng total RNA was used to generate cDNA. The amplification conditions were 95°C for 10 min followed by 40 cycles of 95°C for 15 s, 56°C for 1 min, and 72°C for 1 min, using an Applied Biosystems 7500 Fast instrument. Only one PCR product was obtained for all primers and all samples, based on melting curve analysis. All procedures that involved mice were approved by the University of Vermont Institutional Animal Care and Use Committee.

RESULTS

***plcH* and *pchP* induction in response to choline, GB, and DMG is dependent on the GbdR transcription factor.** In previously published studies, we showed that the GbdR transcription factor controls the expression of *gbcAB* and *dgcAB*, which are required for GB and DMG degradation, respectively, in response to both GB and DMG (43). Because *plcH* expression, PlcH activity, and PchP activity are similarly induced by these two osmoprotectants (18, 36), we hypothesized that GbdR may be the GB- and DMG-dependent regulator of *plcH* and *pchP* induction. To test this hypothesis, PlcH activity was first assessed in Δ*gbdR* mutant cultures by both the NPPC hydrolysis assay and assessing hemolysis on blood agar plates. Cultures of *P. aeruginosa* PAO1 WT, PAO1 Δ*gbdR*, PAO1 Δ*plcHR*, and PAO1 Δ*gbdR* complemented with a functional copy of *gbdR* at the *att* site were grown in T broth with choline, and the supernatants were analyzed for NPPC hydrolysis activity (Fig. 1A). In WT supernatants, the addition of choline to the medium led to a 4.2-fold increase in NPPC hydrolysis activity; no increase in NPPC cleavage activity was observed upon addition of choline in either the Δ*gbdR* or Δ*plcHR* strain. Complementation of the *gbdR* mutant partially restored the induction of PlcH activity in response to choline (Fig. 1A). In the blood agar hemolysis assay, zones of clearing were observed around the WT but not the Δ*plcH* and Δ*gbdR* mutants. Complementation of the Δ*gbdR* mutant restored hemolytic activity (Fig. 1B).

The regulation of PlcH enzyme activity by GbdR suggested the possibility that GbdR regulates the induction of *plcH* transcription. *plcH* transcription has been shown to be induced in response to choline, GB, and DMG (36), and induction by choline is not observed in a *betB* mutant strain which cannot convert choline to GB, indicating that GB and/or its catabolic product DMG is the compound responsible for *plcH* induction in choline-containing media (31). We have shown that either GB or DMG can stimulate GbdR-dependent transcription of the GB catabolic genes even in a mutant (Δ*gbcAB*) that is unable to convert GB to DMG, suggesting that both GB and DMG can serve as inducing compounds (43). To determine if GbdR also regulates *plcH* induction by GB and DMG, we used qRT-PCR to measure *plcH* transcripts in WT and Δ*gbdR* strains (Fig. 1C). *plcH* mRNA levels were assessed in both MOPS minimal medium with pyruvate, in which *plcH* transcript levels are very low, and in the same medium amended with GB. The *plcH* transcript levels are reported as relative changes between uninduced (pyruvate) and induced (GB) conditions. The relative transcript level for each strain was normalized to the *rplU* transcript, which has been shown to remain constant relative to total RNA under a variety of conditions (14). In the presence of GB, the *plcH* transcript was induced 2.4- ± 0.09-fold in the WT (Fig. 1C), while no induction was observed in the Δ*gbdR* strain (0.88- ± 0.06-fold change). Incorporation of *gbdR* at the *att* chromosomal site almost fully complemented the phenotype of the Δ*gbdR* strain (2.09- ± 0.08-fold induction). Induction with DMG showed a similar pattern of *plcH* transcriptional activation, in a *gbdR*-dependent manner (data not shown). Similar results were obtained with *P. aeruginosa* strain PA14, with a 6.69- ± 0.44-fold induction of *plcH* transcript upon induction with GB and no induction (0.64- ± 0.03-fold) in the Δ*gbdR* mutant (Fig. 1D). These data

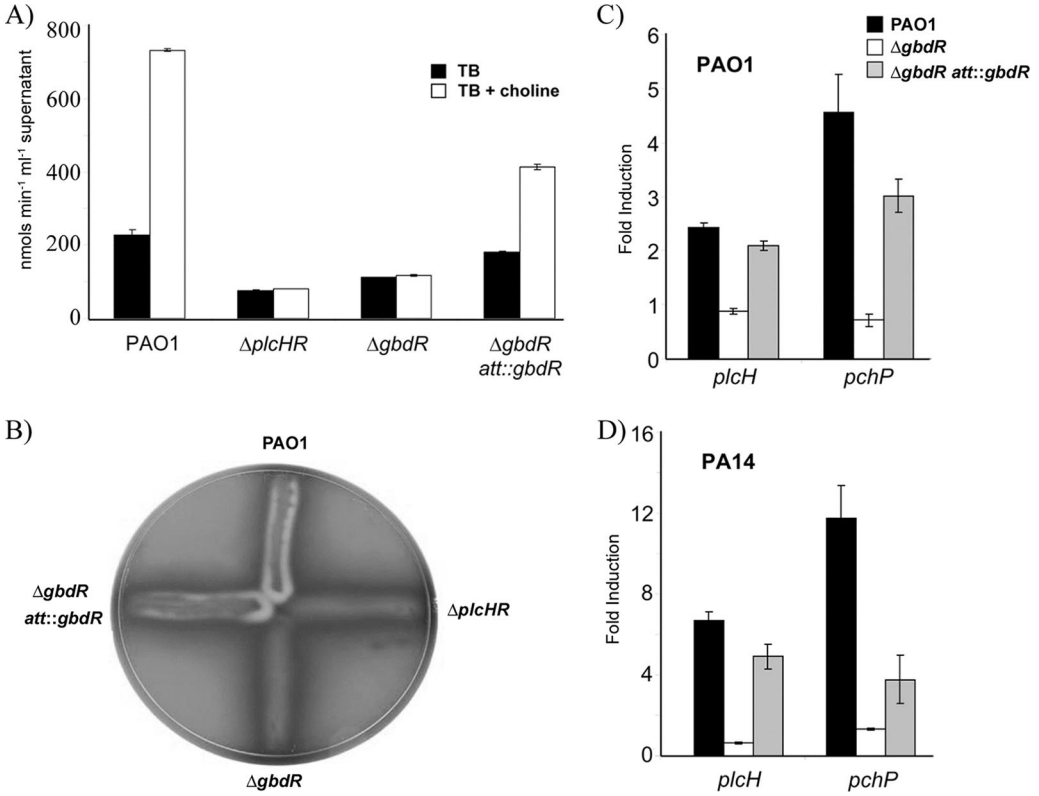

FIG. 1. Role of GbdR transcription factor in controlling PlcH activity and *plcH* transcript levels. (A and B) PAO1 WT, Δ*plcHR* and Δ*gbdR* mutants, and a Δ*gbdR* mutant complemented with *gbdR* at the *att* locus (Δ*gbdR att*::*gbdR*) were assayed for choline-induced NPPC hydrolysis activity in cultures grown in T broth (TB) (black bars) or T broth containing choline (white bars) (A) and in a hemolysis assay using phosphate-supplemented blood agar plates (B). (C and D) qRT-PCR quantification of *plcH* and *pchP* transcript levels normalized to *rplU* levels in *P. aeruginosa* strains PAO1 (C) and PA14 (D). Transcript induction is reported as the transcript level measured under inducing conditions (GB) divided by the transcript level under noninducing conditions (pyruvate). Error bars represent standard deviations between levels in three replicate cultures, and results were comparable between at least three separate experiments.

support the hypothesis that *plcH* transcription is regulated by GbdR.

Phosphorylcholine, a product of PlcH-mediated cleavage of PC and SM, is hydrolyzed by the periplasmic phosphorylcholine phosphatase, PchP, to yield choline. While to our knowledge induction conditions for the *pchP* transcript have not previously been reported in the literature, functional assays have been used to demonstrate induction of enzyme activity in response to choline, GB, and DMG, similar to the regulation observed for *plcH* (18, 20). We hypothesized that *pchP* induction was also dependent on GbdR. Similar to the results with *plcH*, the *pchP* transcript was induced in response to GB compared to pyruvate in PAO1 WT (4.5- ± 0.7-fold) (Fig. 1B) but not in the Δ*gbdR* strain (0.71- ± 0.1-fold). Integration of *gbdR* at the *att* chromosomal site partially rescued the GB-dependent induction (3.1- ± 0.3-fold). A similar pattern of induction of *pchP* by GB in a GbdR-dependent manner was observed in *P. aeruginosa* strain PA14 (Fig. 1D). These data support the regulation of *pchP* by GbdR in a manner similar to that of *plcH*.

Our previous studies with GbdR demonstrated that either GB or DMG could induce GbdR-dependent transcription of GB and DMG catabolic genes, and therefore we hypothesized that both GB and DMG would be capable of inducing *plcH* and *pchP*. To test this, we analyzed *plcH* and *pchP* transcript levels in strains unable to catabolize either compound (43). In a *P. aeruginosa* PAO1 Δ*gbcAB* strain, which is unable to convert GB to DMG, both GB and DMG induced the *plcH* transcript (3.62- ± 0.87-fold and 3.23- ± 0.64-fold, respectively). In a *P. aeruginosa* PAO1 Δ*dgcA* mutant, which is unable to convert DMG to sarcosine, DMG was sufficient to induce *plcH* transcription 2.88- ± 0.35-fold. Together, these data indicate that both GB and DMG are capable of stimulating *plcH* transcription.

Deletion mapping to determine the region necessary for GbdR-dependent induction in the *plcH* and *pchP* promoters. To determine the segment of each promoter that was necessary for GbdR-dependent activation of *plcH* and *pchP* transcription, *plcH* and *pchP* promoter fragments were cloned upstream of the *lacZYA* and *gfp-mut3* reporter genes, respectively (Fig. 2A and B). The resulting constructs were transformed into both WT and Δ*gbdR* backgrounds. Transcriptional activation was assessed in cells grown either in medium with pyruvate as the sole source of carbon or in medium containing pyruvate plus GB. For the full-length *plcH* fragment (plcH-A) and two of the truncated fragments (plcH-B and plcH-C), transcriptional activation was observed in the WT strain but not in the Δ*gbdR* mutant (Fig. 2A). The smallest promoter

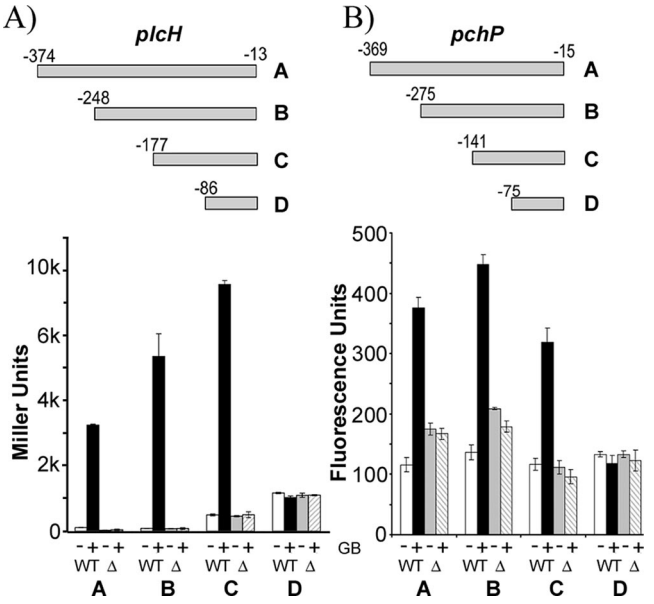

FIG. 2. Promoter truncation analysis of the *plcH* and *pchP* promoters. (A) Schematic map of the truncation constructs for the *plcH* promoter, indicating their positions relative to the translational start site, and their corresponding abilities to induce *lacZ* transcription in medium with GB. The fusion constructs (A to D) were analyzed in the WT and Δ*gbdR* (Δ) strain backgrounds in the absence (−) and presence (+) of GB. (B) Schematic map of the truncation constructs for the *pchP* promoter, indicating their positions relative to the translational start site, and corresponding *gfp* fluorescence driven by the truncated *pchP*-reporter constructs, measured in arbitrary fluorescence units after subtraction of the PAO1 WT (not expressing *gfp*) autofluorescence baseline. Error bars represent standard deviations between levels in three replicate cultures, and results were comparable between at least three separate experiments.

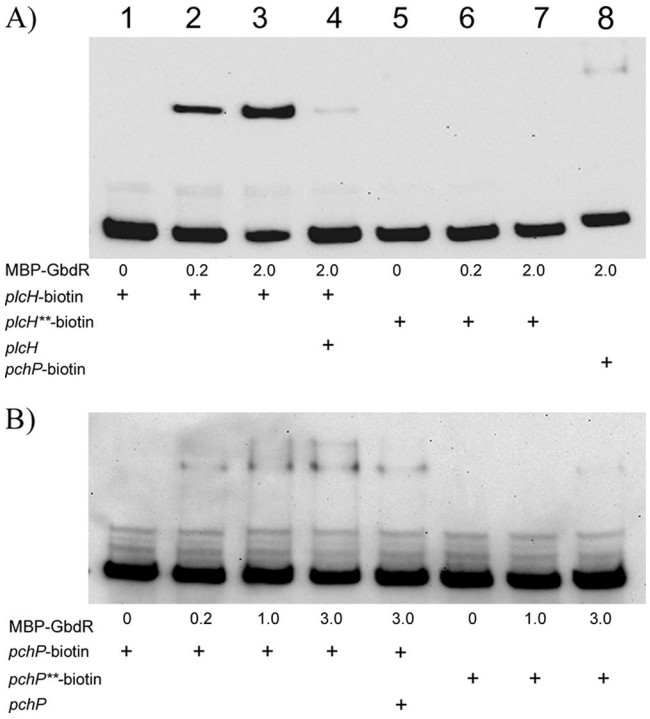

FIG. 3. EMSA to examine interactions between MBP-GbdR and *plcH* and *pchP* promoter fragments. (A) The WT *plcH* promoter fragment was incubated alone or with MBP-GbdR (0.2 and 2 μM) (lanes 1 to 3); to the reaction mix in lane 4, unlabeled *plcH* probe was added in excess. Similar incubations were performed with a probe spanning the same region but with two nucleotide substitutions (*plcH***) (lanes 5 to 7). In lane 8, the shift of the *pchP* promoter probe is shown for comparison. (B) The WT *pchP* promoter probe (lanes 1 to 5) and the mutated promoter probe (*pchP***) (lanes 6 to 8) were incubated with MBP-GbdR at the concentrations shown (μM). An excess of unlabeled *pchP* probe was added to the reaction mix in lane 5.

fragment (plcH-D) failed to show GB-dependent induction (Fig. 2A). This suggests that the promoter region necessary for GB-dependent transcriptional induction is between positions −177 and −86 or overlaps the −86 site.

For *pchP*, a promoter truncation series fused to the *gfp* reporter gene was constructed. The three longest constructs retained GB-dependent induction of green fluorescent protein fluorescence and did not show induction in the absence of *gbdR* (Fig. 2B), while the shortest construct (D) showed no induction by GB. This suggests that nucleotides within the region between −141 and −75 bp are required for GB-dependent transcriptional induction in the *pchP* promoter.

Evidence for GbdR binding to the *plcH* and *pchP* promoters. Our studies above identified the regions of the *plcH* and *pchP* promoters that were necessary for GbdR-dependent induction of reporter activity in response to GB. To test the hypothesis that GbdR interacts directly with the *plcH* promoter, we used a 164-bp fragment of the *plcH* promoter (corresponding to fragment C from the promoter deletion analysis) in an EMSA. GbdR was purified as an MBP fusion (MBP-GbdR); the MBP-GbdR expression construct was sufficient to restore growth on GB in the Δ*gbdR* mutant, indicating that the fusion protein was functional. MBP-GbdR specifically shifted the *plcH* promoter with increasing concentrations of protein (Fig. 3A, lanes 2 and 3). This shift could be competed with unlabeled *plcH* promoter (Fig. 3A, lane 4). We also tested whether MBP-GbdR binds to the *pchP* promoter by using a 260-bp fragment of the *pchP* promoter (corresponding to fragment B of our promoter deletion analysis). MBP-GbdR specifically shifted the *pchP* promoter with increasing concentrations of protein, leading to a larger proportion of the fragment shifting and to an additional shifted band (Fig. 3B, lanes 2 to 4), although the efficiency of binding was lower than that for *plcH* (Fig. 3A, lane 8). This shift could be competed with unlabeled *pchP* promoter (Fig. 3B, lane 5). The lower affinity of our MBP-GbdR fusion for the *pchP* promoter under these conditions is consistent with our promoter fusion data showing that the induction of the *pchP* transcript is less than that seen with the *plcH* promoter. The MBP-GbdR protein bound to both the *plcH* and *pchP* promoters in the presence (Fig. 3A) and absence (data not shown) of GB. Combined with the promoter deletion analyses and the induction studies, this in vitro binding is strong support for direct GbdR binding and regulation at the *plcH* and *pchP* promoters.

Identification and mutation of conserved residues in the *plcH* and *pchP* promoters that are necessary for GbdR induction. The promoter regions necessary for *plcH* and *pchP* induction by GB were aligned with each other by use of LALIGN

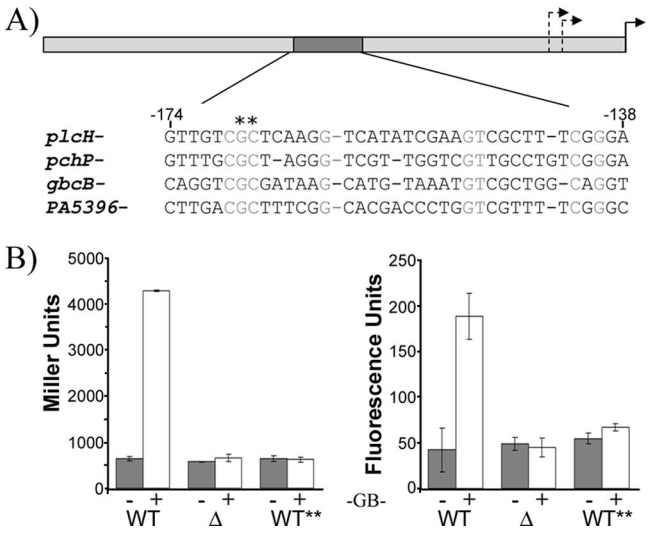

FIG. 4. Role of two conserved base pairs in the *plcH* and *pchP* promoters in GbdR-dependent induction. (A) Schematic of the *plcH* promoter showing the conserved promoter region, with base pairs denoting the distance from the translational start site. The phosphate- and choline-dependent transcriptional start sites are shown with dotted arrows (41). The alignment shows a common promoter region present near four genes controlled by GbdR. The asterisks mark the two base pairs changed to adenines in the mutant constructs. (B) The nonmutated promoter fusions were analyzed in the WT and Δ*gbdR* (Δ) strain backgrounds in the presence (+) and absence (−) of GB. The mutated constructs with the 2-bp alteration were analyzed in the WT background (WT**). Error bars represent standard deviations between levels in three replicate cultures, and results were comparable between at least three separate experiments.

(11). One region of alignment corresponding to positions −174 to −138 of the *plcH* promoter was also aligned via the LALIGN pairwise method to the promoter regions of other genes known to be controlled by GbdR, including *gbcB*, involved in GB catabolism, and PA5396, the first gene in the putative operon that contains the DMG catabolic genes. The alignment of these promoters is shown in Fig. 4A. The transcriptional start sites for the phosphate- and choline-dependent *plcH* transcripts, as demonstrated by Vasil and coworkers (41), are shown with dotted arrows (Fig. 4A).

To test the hypothesis that the aligned regions represented the motif recognized by GbdR, we mutated GC residues (marked with asterisks in Fig. 2) to adenines in both the *plcH* and *pchP* promoters. In both cases, promoter fusion constructs that were identical to plcH-C and pchP-C (Fig. 2A and B), respectively, except for this 2-bp mutation, completely lacked GbdR-dependent induction of transcription in medium with GB (Fig. 4B). The 2-bp changes made in the *plcH* and *pchP* promoters were not associated with either the −10/−35 sites or the ribosome binding site, consistent with the observation that basal transcription of *plcH* or *pchP* in strains carrying the mutated promoters is not different from that in strains carrying the nonmutated versions (Fig. 4B). Mutation of the adjacent C residue at −169 to an adenine in the *plcH* promoter led to abrogation of all GbdR-dependent induction, similar to the mutations described above, while mutation of the G residue at −161 led to a 59% decrease in induction of GbdR-dependent transcription levels (data not shown).

To determine if the conserved promoter residues represented part of the region required for GbdR binding to the promoters, we tested the ability of either the mutated *plcH* (*plcH***) or *pchP* (*pchP***) promoter to bind MBP-GbdR in an EMSA (Fig. 3A and B). The *plcH*** mutant promoter (Fig. 3A, lanes 5 to 7) failed to show a shift at any tested concentration of MBP-GbdR. The mutant *pchP* promoter (*pchP***) could be shifted by the addition of 3.0 μM MBP-GbdR, but at a much lower affinity than the WT promoter (Fig. 3B, lanes 6 to 8). The defects of these mutant promoter fragments in associating with MBP-GbdR suggest that these two base pairs are important for GbdR binding.

GbdR controls *plcH* and *pchP* induction in response to surfactant and BALF and in the mouse lung. The major component of mammalian lung surfactant is PC. As such, this compound represents a sizable pool of PlcH substrate within the lung. To test whether GbdR controls *plcH* and *pchP* gene induction in response to surfactant, we grew PAO1 WT and Δ*gbdR* strains in MOPS medium containing 10 mM pyruvate or the same medium amended with the bovine-derived surfactant Survanta. RNAs were harvested from cells, and qRT-PCR was used to determine *plcH*, *pchP*, and *rplU* transcript levels; *plcH* and *pchP* levels were normalized to levels of *rplU*. As shown in Fig. 5A, *P. aeruginosa* WT induced *plcH* and *pchP* 6.4-fold and 8.3-fold, respectively, in the presence of Survanta over levels in medium without surfactant addition. No induction was observed in the Δ*gbdR* mutant. Similar induction experiments were performed with mouse BALF. *P. aeruginosa* strains were resuspended in sterile filtered BALF or DPBS, with 10 mM pyruvate added to both. In BALF-pyruvate, the normalized transcript levels of *plcH* and *pchP* were 10.2- and 4.6-fold higher, respectively, than levels in cells incubated in buffer with pyruvate. No induction upon incubation in BALF was observed for the Δ*gbdR* strain (Fig. 5B).

To test the importance of GbdR for the regulation of *plcH* and *pchP* in *P. aeruginosa* within the mouse lung, we infected C57BL/6 mice with 1×10^8 CFU of either PAO1 or the Δ*gbdR* mutant via oropharyngeal aspiration ($n = 7$ per group). After 24 h, the BALF was harvested and total RNA isolated and analyzed by quantitative PCR. *plcH* and *pchP* transcript signals were normalized to that of *rplU*. The results of the transcript analyses are shown in Fig. 5C. Both *plcH* and *pchP* showed significantly higher transcript levels in WT cells than in Δ*gbdR* cells. These data support an important role for GbdR in regulation of *plcH* and *pchP* in vivo.

Examination of the *plcH* positive feedback induction loop. Based on the work presented here and data and interpretations published by others (8, 41), we hypothesize that PlcH activity initiates a series of catalytic steps leading to GbdR-dependent induction of *plcH* and *pchP*, establishing a positive feedback loop in which choline phosphate is degraded to GB, which further induces *plcH* expression (Fig. 6A). To establish the role of each predicted member of the proposed positive feedback loop, we measured the expression of the *plcH* promoter fusion in the PAO1 WT and in mutant strains, including the Δ*plcHR*, *pchP*::Tn, *betB*::Tn, and Δ*gbdR* strains, in the presence of MOPS-Survanta medium (containing approximately 0.25 mg/ml PC) (Fig. 6B). As shown above (Fig. 5A), *plcH* and *pchP* transcription in this medium was induced in the WT but not in the *gbdR* mutant. There was no induction of the *plcH*

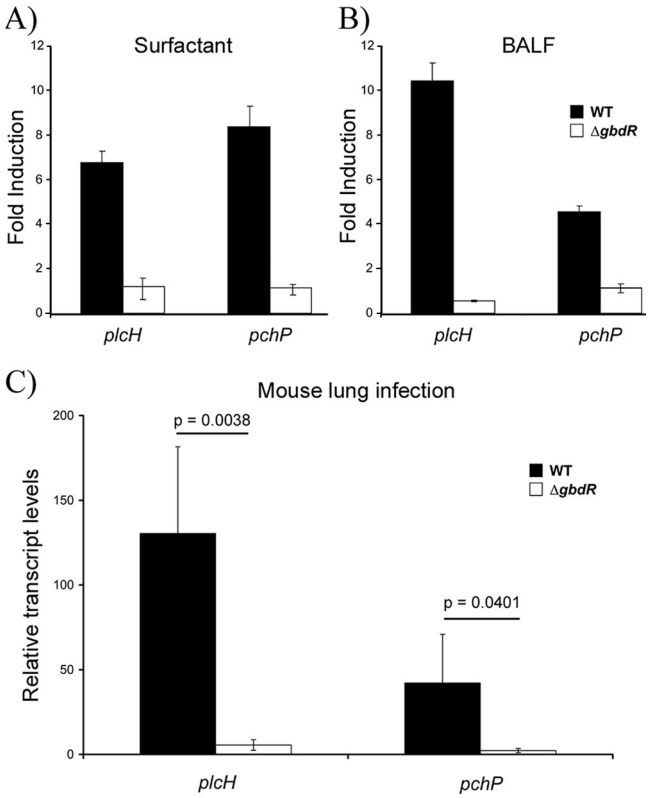

FIG. 5. GbdR-dependent regulation of *plcH* and *pchP* transcripts in Survanta and murine BALF and during mouse lung infection. (A) RNAs from WT (black bars) and Δ*gbdR* (white bars) cells grown in medium with and without surfactant were analyzed by qRT-PCR to determine relative *plcH* and *pchP* transcript levels; transcript levels were normalized to those of the *rplU* control transcript. The levels of induction represent transcript levels in cells grown in medium with Survanta divided by levels in cells grown with pyruvate as the sole source of carbon. (B) qRT-PCR with cells grown in filter-sterilized murine BALF from uninfected mice. Induction is reported as in panel A. (C) qRT-PCR was conducted on RNAs isolated from BALF of mice infected with either PAO1 WT or PAO1 Δ*gbdR* for 24 h. Transcript levels are reported as *plcH* or *pchP* transcript levels relative to levels of the *rplU* transcript. There were seven mice infected for each group, and standard errors are shown.

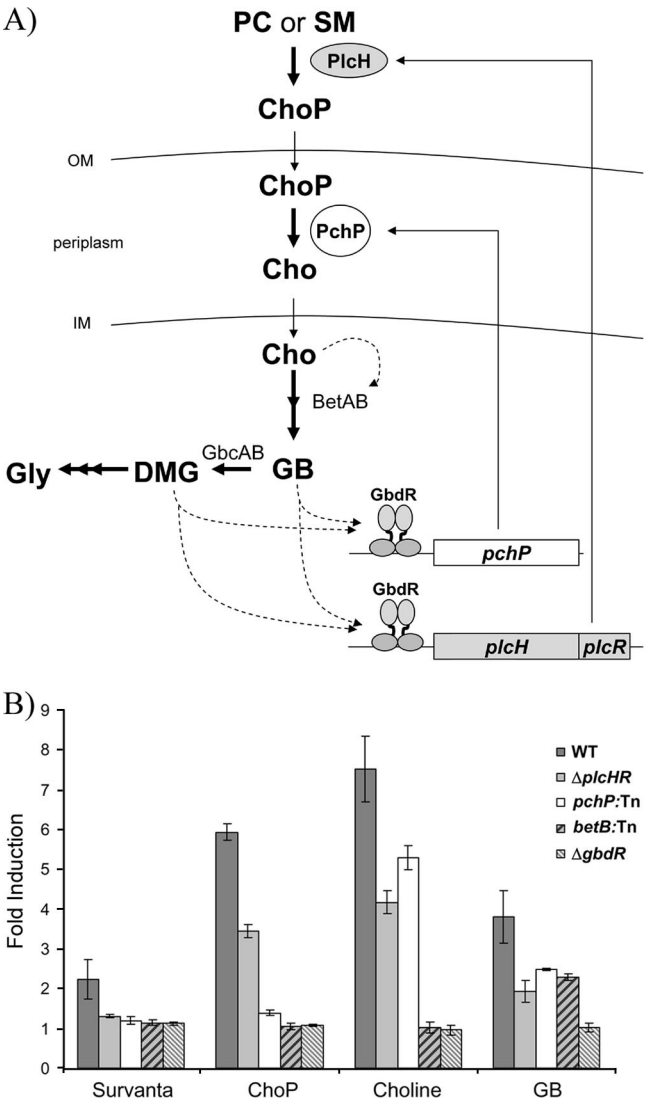

FIG. 6. PlcH is the initiator of a positive feedback loop during *P. aeruginosa* exposure to lung surfactant (Survanta). (A) Model for the GbdR-dependent, GB-induced PlcH positive feedback loop. Thick solid arrows represent catalytic activity conducted by the adjacent protein, and thin solid arrows represent transport events. Dashed arrows represent induction events. (B) PAO1 WT and mutants defective in *plcH*, *pchP*, *betB*, and *gbdR* carrying the pMW22 *plcH* reporter construct were exposed to MOPS medium with diluted Survanta (1%). Similar cultures were amended with phosphorylcholine (ChoP), choline, or GB to a final concentration of 0.25 mM. Induction is reported as the Miller units for the listed induction conditions divided by the Miller units for comparable cultures grown in MOPS-pyruvate medium. Error bars represent standard deviations between levels in three replicate cultures, and results are comparable between at least three separate experiments.

transcriptional fusion in the *plcH*, *pchP*, or *betB* mutant, suggesting that PC catabolism was necessary for induction of *plcH* by GbdR (Fig. 6B). To explore the role of choline phosphate (ChoP), choline, and GB in the pathway, we added 0.25 mM of each compound to the medium and assessed *plcH* transcription activity. The addition of ChoP resulted in *plcH* induction in a Δ*plcHR* strain but not in the *pchP*::Tn or *betB*::Tn strain, underlining the importance of the PchP phosphatase in ChoP hydrolysis and resultant choline catabolite-dependent gene induction. The addition of choline restored the induction of *plcH* in the Δ*plcHR* and *pchP*::Tn strains, but choline was unable to induce the *plcH* promoter fusion in the *betB*::Tn strain, as reported previously (31). GB restored *plcH* induction in all strains but the Δ*gbdR* mutant. These data highlight the steps critical for *plcH* induction in lung surfactant as well as establish the role of each enzyme in the initiation of the *plcH* positive feedback induction system.

DISCUSSION

In this study, we identified GbdR as the transcription factor required for induction of the *plcH* and *pchP* transcripts in the presence of GB and DMG. Using promoter deletion and mutation analysis, we identified residues required for GbdR-dependent induction and demonstrated direct binding to the *plcH* and *pchP* promoters by EMSA. GbdR is required for

induction of *plcH* and *pchP* in bovine lung surfactant and mouse BALF. In addition, using an acute model of mouse lung infection, we showed that GbdR plays an important role in induction of *plcH* and *pchP* in vivo.

The two known stimuli of *plcH* regulation, low phosphate and the presence of GB, regulate *plcH* in different ways. Transcriptional induction of the *plcH* gene in response to limiting phosphate conditions is controlled by PhoB and is hypothesized to function as part of a phosphate-scavenging system (36). The acquisition of phosphate from PC and SM will eventually turn off *plcH* induction via the Pho system. The regulation of *plcH* transcription by GbdR in response to GB represents a positive feedback regulatory scheme. As PlcH activity releases more choline from PC and SM, conversion of choline to GB will lead to induction of *plcH* transcription by GbdR. By adding each metabolite in the proposed *plcH* induction pathway (Fig. 6A) to strains capable or incapable of each predicted catabolic step, we were able to fully validate the role of each intermediate in the PlcH-initiated positive feedback loop during *P. aeruginosa* interaction with host-derived phospholipids (Fig. 6B). The positive feedback regulation, leading to increased PlcH production and increased host damage, may in part explain the striking phenotypes of *plcH* mutants in a variety of animal model systems (12, 27, 29, 46).

While *P. aeruginosa* produces multiple PLCs and phosphatases, PlcH and PchP appear to be of major importance for inducing GbdR induction of virulence-regulated genes (2, 26). In PC-rich Survanta, experiments using the *plcH* reporter system supported the hypothesis that PlcH is critical for ChoP acquisition (Fig. 6B), suggesting that other described PLCs (2, 26) do not play a significant role in PC hydrolysis under these conditions. The phosphorylcholine phosphatase (PchP) was identified biochemically and subsequently cloned (18, 20). We are unaware of any published reports that verify the requirement of PchP for hydrolysis of ChoP in an environment similar to that used in our assay. Although a number of other putative phosphatases are produced by *P. aeruginosa*, some of which are highly induced during growth on Survanta (M. J. Wargo and D. A. Hogan, unpublished data), we established that, in the time frame of our assay, PchP is required for *plcH* induction in Survanta (Fig. 6B).

plcH transcript accumulation is likely impacted by many different factors. Because there are several catabolic steps involved in generating GB for induction of *plcH* by GbdR in PC- and SM-rich environments such as the lung, induction of *plcH* by GB and DMG could be regulated at a number of points. In particular, while we have discussed PlcH as a single enzyme for the sake of simplicity, PlcH can be present freely or in complex with $PlcR_1$ or $PlcR_2$, two chaperones encoded by overlapping reading frames of the *plcR* gene (6). These complexes have different biochemical activities from that of the free enzyme (17). Interestingly, it has been shown that choline induction fails to robustly induce the *plcR* transcript, while phosphate starvation is capable of inducing both *plcH* and *plcR* (17). *plcH* transcript stability can also vary in response to iron levels (25). The availability of the choline-derived GB-inducing signal could also be affected by choline phosphatase activity, choline uptake, or choline conversion of choline into GB. *betAB* gene induction by choline is independent of GbdR (30, 42, 43). Previous work has shown only very slight alterations (<2-fold changes) in *gbdR* transcript levels upon growth in media containing choline, GB, or DMG (45); mechanisms for regulating GbdR activity have not been explored. Due to the complexity of the *plcH* positive feedback loop, there are a variety of opportunities for the development of strategies for the inhibition of this pathway in vivo.

Although these analyses elucidate the major GB- and DMG-dependent regulator of *plcH* and *pchP* and show the role of GbdR during lung infection, several questions remain to be answered. First, it is important to understand how the phosphate and GB/DMG signals are integrated at both the *plcH* and *pchP* promoters by assessing the interaction between GbdR and PhoB. Second, understanding the mechanism of catabolite repression (34) of these loci and other genes related to GB and DMG catabolism will enable us to better understand the regulation of virulence factors by cellular metabolic processes. Finally, understanding the intricacies of GbdR-dependent regulation in *P. aeruginosa* during infection could shed light on the respective roles of GB utilization as a carbon and nitrogen source, an osmoprotectant (38, 44), and an inducer of virulence-related genes such as *plcH* in different environments.

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