Phosphoglycerate Kinase Is Involved in Carbohydrate Utilization, Extracellular Polysaccharide Biosynthesis, and Cell Motility of Xanthomonas axonopodis pv. glycines Independent of Clp

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Phosphoglycerate kinase (Pgk), catalyzing the reversible conversions between glycerate-1.3-2P and glycerate-3P, plays an important role in carbohydrate metabolism. Here, we show that a Pgk-deficient mutant (NΔpgk) of Xanthomonas axonopodis pv. glycines (Xag) could grow in medium with glucose, galactose, fructose, mannose, or sucrose, as the sole carbon source, suggesting that Xag may employ Entner-Doudoroff (ED) and pentose phosphate pathway (PPP), but not glycolysis, to catabolize glucose. NΔpgk could not utilize pyruvate, suggesting that Pgk might be essential for gluconeogenesis. Mutation in pgk led to a reduction of extracellular polysaccharide (EPS) biosynthesis, cell motility, and intracellular ATP. As a result, the virulence of NΔpgk was significantly compromised in soybean. NΔpgk could be fully complemented by the wild-type pgk, but not by clp (encoding Crp-like protein). qRT-PCR analyses demonstrated that pgk is regulated by the HrpG/HrpX cascade, but not by Clp. These results suggest that Pgk is involved in carbohydrate utilization, EPS biosynthesis, and cell motility of Xag independent of Clp.

Keywords: Xanthomonas axonopodis pv. glycines, phosphoglycerate kinase, Crp-like protein, carbohydrate utilization, extracellular polysaccharide, cell motility

INTRODUCTION

Xanthomonas axonopodis pv. glycines (Xag) is widely distributed in soybean-producing areas throughout the world (Chatnaparat et al., 2012). The pathogen enters soybean leaves through stomata or wounds, and reproduces within the intercellular spaces of the spongy mesophyll, resulting in erumpent pustules surrounded by yellow halos (Guo et al., 2019). Xag is a quarantine pathogen that can be spread by rain droplets during the growing season and by seed transportation (Liu et al., 2016). Bacterial pustule, caused by Xag, is an important bacterial disease on soybean, resulting in premature defoliation and decreasing yield and seed quality (Thowthampitak et al., 2008; Athinuwat et al., 2009).
Over the past two decades, significant progress has been made on elucidating the association of carbon metabolism to virulence and quorum sensing (QS) in *Xanthomonas* spp., such as in *X. oryzae* pv. *oryzicola* (Xoc), which infects rice (Guo et al., 2017). Results of these studies have shown that carbohydrate acquisition is essential for the pathogen to grow and establish a successful infection within the hosts (Tang et al., 2005; Mellgren et al., 2009). When the pathogen gains entry into the host plant, it first propagates in the apoplastic space until reaching a density threshold, followed by the expression of lots of virulence-related genes and secretion of cell-wall degrading enzymes through the diffusible signal factor (DSF)-mediated QS mechanism (Büttner and Bonas, 2010). The pathogen overcomes host defenses, adapts to the hostile environment, and ultimately degrades the plant parenchyma cells to gain access to its nutritional reservoirs (Tamir-Ariel et al., 2007; Zhou et al., 2013).

*Xanthomonas* spp. carry out catabolic processes, such as glycolysis, Entner-Doudoroff (ED), pentose phosphate pathway (PPP), and the tricarboxylic acid (TCA) cycle, to catabolize glucose and other simple sugars (sucrose, fructose, mannose, and galactose) (Lu et al., 2009; Kim et al., 2010). These metabolic pathways provide nutrition for growth and propagation of pathogen and supply energy that drives energy-requiring activities such as extracellular polysaccharide (EPS) production and cell motility (Mellgren et al., 2009; Guo et al., 2017). Phosphoglycerate kinase (Pgk), reversibly converting glycerate-1,3-2P to glycerate-3P, is involved in glycolysis, ED, PPP, and gluconeogenesis in Xag. However, little is known about the biological processes in which Pgk participates.

Acetyl-CoA and amino acids, produced by catabolic processes, act as precursor molecules for synthesizing DSF signals (Zhou et al., 2015). Crp-like protein (Clp), located at the end of the DSF signaling pathway, is the global regulator in *Xanthomonas* spp. In addition to DSF signals, Clp could also converge on low-oxygen signals transduced by the RavS/RavR two-component regulatory system (He et al., 2009) and environmental signals transduced by the HrpG/HrpX cascade (Wengelnik and Bonas, 1996; Wengelnik et al., 1996). As a consequence, the activated Clp directly or indirectly regulates the expression of downstream genes and modulates numerous biological properties, such as carbohydrate utilization, cell motility, EPS production, and synthesis of extracellular enzymes (Guo et al., 2019), which are all essential for virulence and adaptation of *Xanthomonas* spp. in host plant. However, it is unknown whether Clp could completely or partially complement the impaired properties in the *pgk* mutant. In addition, the inherent relationship between Clp and Pgk is unclear.

In this study, we aimed to explore the biological properties of Pgk and to reveal its association with Clp. Here, we present evidence that Pgk is involved in carbohydrate utilization, cell motility, and EPS biosynthesis independent of Clp in Xag. In addition, the expression of *pgk* is regulated by the HrpG/HrpX cascade, but not by DSF signals or Clp.

**MATERIALS AND METHODS**

**Bacterial Strains, Plasmids, and Culture Conditions**

Strains and plasmids used in this study are listed in [Supplementary Table S1](#). Unless otherwise specified, *Xag* NEAU001 and its derivative strains were grown at 28°C in NYG (5 g L\(^{-1}\) polypeptone, 3 g L\(^{-1}\) yeast extract, 20 g L\(^{-1}\) glycerol), NY (NYG without glycerol), or NCM (2 g L\(^{-1}\) (NH\(_4\))\(_2\)SO\(_4\), 0.2 g L\(^{-1}\) MgSO\(_4\)·7H\(_2\)O, 4 g L\(^{-1}\) K\(_2\)HPO\(_4\), 6 g L\(^{-1}\) KH\(_2\)PO\(_4\)) media (Liu et al., 2019). *Escherichia coli* strains were routinely grown at 37°C in LB (10 g L\(^{-1}\) tryptone, 10 g L\(^{-1}\) NaCl, 5 g L\(^{-1}\) yeast extract) medium. Antibiotics were added at the following concentrations: ampicillin, 50 µg mL\(^{-1}\); kanamycin, 25 µg mL\(^{-1}\); spectinomycin, 100 µg mL\(^{-1}\); and carbenicillin, 50 µg mL\(^{-1}\).

**Generation of the pgk Deletion Mutant, Complementation, and By-Path Complementation Strains**

The in-frame deletion mutant of *pgk* in *Xag* was generated by using homologs recombination as described by Li et al. (2011), using pKMS1 as a suicide vector and the primers listed in [Supplementary Table S2](#). The obtained mutant was named N\(_{Δpgk}\). The complemented and by-path complemented strains were also constructed by separately introducing the recombinant plasmids pCpgk and pCclp into N\(_{Δpgk}\) (Li et al., 2011). The resulting complemented and by-path complemented strains were designated as C\(_{Δpgk}\) and N\(_{Δpgk(clp)}\), respectively.

**Qualitative/Quantitative Analysis of Carbohydrate Utilization and EPS Production**

Carbohydrate utilization and EPS production assays were measured as previously described by Lu et al. (2009). Each experiment was repeated at least in triplicate.

**Determination of Cell Motility and Exoenzyme Activity**

Cell motility of *Xag* strains was investigated on plates of semi-solid NY medium with 0.3% agar as described by Long et al. (2018). The exoenzyme activities of *Xag* strains in the supernatants of the cultures were analyzed on NY plates supplemented with different substrates (such as skimmed milk for protease, carboxymethyl cellulose for carboxymethylcellulase, soluble starch for α-amylase, and locust bean gum for endo-β-mannanase) following the method described previously by Thowthampitak et al. (2008). The experiment was repeated independently in triplicate, and five replicate plates were conducted for each treatment.

**Determination of Intracellular ATP**

Intracellular ATP levels were determined by using an ATP Assay Kit (Beyotime), following the manufacturer’s protocols. All samples were measured at least in triplicate.
Sensitivity to Hydrogen Peroxide (H$_2$O$_2$)

H$_2$O$_2$ resistance assays were performed as described previously by Wang et al. (2013). Briefly, the fresh Xag strains were grown in NY medium at 28°C until the exponential growth phase was reached (OD$_{600}$ ≈ 1.0). Then, the semi-solid NY plates mixed with Xag were prepared. After solidification, the saturated filter papers with 0.5 or 1.0 mM H$_2$O$_2$ were placed on the center of the plates. These bioassay plates were then incubated at 28°C for 2 days. H$_2$O$_2$ resistance was indicated by measuring the diameter of the zone of inhibition. The same experiment was repeated at least three times.

qRT-PCR

In addition to pgk-related strains, the following strains were used in the qRT-PCR: the deletion mutants of rpfF (responsible for the synthesis of DSF), rpfC, rpfG (the RpfC/RpfG two-component system is involved in sensing and transduction of DSF) (Cai et al., 2017), rpfB (required for DSF signals turnover) (Wang et al., 2016), rpfS (the RpfS/RpfR two-component system is involved in sensing and transduction of low-oxygen signals) (He et al., 2009); and the deletion mutants of hrpX, hrpG, and their upstream regulatory genes, such as rsmA (Andrade et al., 2014), zur (Huang et al., 2009), trh (Li et al., 2011), and xopL (also called hrpX) (Islam et al., 2009), which are all involved in regulating the mRNA level of clp in Xag (Guo et al., 2019). All tested strains were grown in NY medium until OD$_{600}$ ≈ 2.0. Total RNA was extracted using the Trizol reagent (Invitrogen), following the manufacturer’s protocol. cDNA synthesis was conducted with a PrimeScript™ RT reagent Kit (TaKaRa). The transcriptional levels of tested genes were determined by qRT-PCR using the primers listed in Supplementary Table S2. qRT-PCR was performed on the Applied Biosystems™ 7500 Real-Time PCR System using SYBR Premix ExTaq™ (TaKaRa). The transcriptional level of integration host factor A (ihaA) was used as a reference (Thowthampitak et al., 2008). All qRT-PCR analyses were performed in two independent experiments.

Plant Assays

The virulence of Xag strains was assessed as described previously (Chatnaparat et al., 2012). Briefly, all tested strains were grown in NY medium with shaking until the exponential phase was reached. Bacterial cells were then harvested, washed twice, and re-suspended to OD$_{600}$ ≈ 0.2. Then bacterial cells were high-pressure sprayed into soybean leaves (Glycine max cv. Zhoudou63; susceptible to bacterial pustule, 6 weeks old). Phenotypes were scored 15 days post-inoculation. The experiments were repeated at least in triplicate.

Bacterial cell suspensions at OD$_{600}$ ≈ 0.1 were infiltrated into the intercellular spaces of leaves with needleless syringes. Bacterial growth within soybean leaves was assessed as previously described by Liu et al. (2014). The experiments were repeated at least in triplicate.

Statistical Analysis

Means and standard deviations (SD) of experimental data were calculated using Microsoft Office Excel. Statistical analyses were performed using a Student’s t-test. *Indicates significance at P < 0.05; **indicate significance at P < 0.01.

RESULTS

Deletion Mutant of pgk From X. axonopodis pv. glycines

Genomic sequence analysis showed that only one open reading frame, in the genome of Xag strain NEAU001, is annotated to encode Pgk. To facilitate the functional study of pgk, a non-polar deletion mutant was constructed by using homologous recombination and pKMS1 as a suicide vector. As expected, a 2,095 bp fragment was amplified from the wild-type Xag strain with the primer pairs 1F/2R, whereas only a 923 bp fragment was amplified in the pgk mutant because of an in-frame deletion of 1,172 bp from pgk (Figure 1). The in-frame deletion was further verified by a nested PCR using the primer pairs 3F/3R (Figure 1). The deletion mutant, designated NΔpgk, was used in our studies.

Pgk Is Involved in the Utilization of Carbohydrates in Xag Independent of Clp

Phosphoglycerate kinase is an indispensable component of glycolysis, ED, PPP, and gluconeogenesis (Kim et al., 2010); therefore, we measured the growth of NΔpgk to determine whether Pgk is involved in carbohydrate metabolism in Xag. We found that NΔpgk has a similar growth pattern to that of the wild-type Xag when grown in NY medium (Supplementary Figure S1), indicating that NΔpgk was not auxotrophic. To qualitatively assess the effect of Pgk on carbohydrate utilization, we used NCM medium, which is similar to the conditions encountered in the plant apoplast, to investigate the growth of NΔpgk. When glucose, galactose, fructose, mannose, sucrose, or pyruvate was used as the sole carbon source, the pgk mutant displayed smaller colonies than the wild-type strain (Supplementary Figure S2), suggesting that mutation in pgk diminishes the ability of Xag to utilize carbohydrates.

To quantitatively evaluate the contribution of Pgk to carbohydrate utilization, the growth of NΔpgk, the complemented strain CNΔpgk, the by-path complemented strain NΔpgk(clp), and the wild-type strain was tested in NCM liquid medium supplemented with different carbon sources. When glucose, galactose, fructose, mannose, or sucrose was used as the sole carbon source, NΔpgk exhibited significantly slower growth compared to the wild-type strain (P < 0.01), and NΔpgk harboring the pgk in trans completely restored the ability to acquire these sugars (Figure 2). Compared with the growth of NΔpgk, the growth rate of NΔpgk(clp) was lower (Figure 2), indicating a reduced ability to acquire sugars supplemented and an optimal level of Clp is required for Xag to utilize carbohydrates. When pyruvate was used as the sole carbon source, the growths of both NΔpgk and NΔpgk(clp) were
FIGURE 1 | Schematic map and molecular analysis of pgk mutation in X. axonopodis pv. glycines (Xag). The positions and orientations of pgk, encoding phosphoglycerate kinase (Pgk), and other adjacent open reading frames (ORFs) are shown using the genome sequence of Xag 12-2 strain as a reference. Numbers and arrows respectively represent the locations and orientations of the ORFs; lines indicate intergenic sequences. A non-polar construction of the pgk deletion mutant was sketched. The gray and brown box indicated where the left and right flanks targeted pgk. The white box shows the 1,172 bp deletion of pgk.

Pgk Is Involved in EPS Biosynthesis of Xag Independent of Clp

Because Pgk is essential for glycolysis, ED, PPP, and gluconeogenesis (Kim et al., 2010), we further explored whether a mutation in pgk has any effect on EPS biosynthesis in Xag. All tested strains were grown on NY plates supplemented with 2% glucose, galactose, fructose, mannose, sucrose, or pyruvate. NΔpgk had smaller colonies than the wild-type strain on all plates tested (Figure 4A), indicating that Pgk might be involved in the biosynthesis of EPS in Xag.

The contribution of Pgk to EPS production was quantitatively examined by culturing all strains in NY liquid medium supplemented with 2% carbohydrates for 5 days. NΔpgk produced approximately 35–85% less EPS than the wild-type when cultured in glucose-, galactose-, fructose-, mannose-, sucrose-, or pyruvate-containing medium (Figure 4B). In addition, the EPS yield of CNΔpgk was fully restored to the wild-type level (Figure 4B). These results demonstrate that Pgk is involved in the biosynthesis of EPS in Xag.

We next determined whether Clp could, either completely or partially, restore EPS production in NΔpgk. Our results showed that by-path complementation of clp in NΔpgk did not restore EPS biosynthesis, on the contrary, it showed weakened EPS biosynthesis (Figures 4A,B). To further understand how Pgk is involved in the biosynthesis of EPS in Xag, qRT-PCRs were performed to explore the transcriptional levels of gum and xan genes. As shown in Figure 4C, the expression of 12 gum genes in NΔpgk was significantly lower than in CNΔpgk and the wild-type strain, indicating that the mutation in pgk negatively regulates the expression of gum genes. The transcriptional levels of gumB and gumC in NΔpgk(clp) was significantly lower than that in the wild-type strain, while the transcriptional levels of
FIGURE 2 | Phosphoglycerate kinase is involved in the utilization of glucose (A), mannose (B), sucrose (C), galactose (D), fructose (E), and pyruvate (F) in Xag independent of Clp. Aliquots were taken in triplicate at intervals of 144 h after incubation at 28 °C, and bacterial growth was determined by measuring OD_{600} against the medium blank. Data are means ± standard deviations (SD) from three repeats.

**gumD-M** in NΔpgk(clp) was significantly higher than that in the wild-type strain, suggesting that by-path complementation of clp up-regulates the expression of **gum** genes except for **gumB** and **gumC**, which play a critical role in the biosynthesis of EPS in Xag. However, a minimal change in the expression level of xan genes was observed in NΔpgk or NΔpgk(clp) (Supplementary Figure S3). These results demonstrate that Pgk is involved in the biosynthesis of EPS in Xag independent of Clp, possibly through partially inhibiting the expression of the **gum** gene cluster.

**Pgk Is Involved in Cell Motility of Xag Independent of Clp**

We explored whether a mutation in pgk affects the cell motility of Xag. Our results showed that there was little visible difference in cell motility among NΔpgk, CNΔpgk, NΔpgk(clp), and the wild-type strain on NY plates (Figure 5A). However, on NY plates supplemented with 0.5% glucose, NΔpgk displayed severely reduced cell motility compared with the wild-type strain (Figure 5A). The diameter of the colonies resulting from migration away from the inoculation sites on the surface of the plate was approximately 0.72 cm for NΔpgk, 2.83 cm for CNΔpgk, 1.21 cm for NΔpgk(clp), and 2.74 cm for the wild-type strain (Figure 5B). A t-test indicated that the mean diameter of NΔpgk was significantly smaller than that of the wild-type strain (P < 0.01), and the cell motility of CNΔpgk was fully restored to the wild-type level.

We then investigated whether Clp could, either partially or completely, restore cell motility of NΔpgk. Interestingly,
by-path complementation of clp in NΔpgk only slightly enhanced the cell motility of NΔpgk compared to that of the wild-type (Figure 5B). To further understand how Pgk is involved in cell motility, qRT-PCRs were performed to explore the expression of fliA, fleN, flhF, rpoN2, and fleQ, which have been shown crucial for cell motility (Guo et al., 2019). As shown in Figure 5C, no change in the expression of these five genes was observed in NΔpgk, CNΔpgk, or the wild-type strain. However, the transcription of these five genes, especially fliA and fleN, was significantly increased in NΔpgk(clp) (P < 0.01) (Figure 5C). As reported previously, the overexpression of clp in the wild-type background also results in the increased expressions of these five genes and slightly enhanced cell motility compared to the wild-type (Guo et al., 2019). Therefore, our results suggest that the by-path complementation of clp could promote cell motility, rather than restore the impaired motility of NΔpgk. Further, Pgk is involved in the cell motility of Xag independent of Clp.

**Pgk Is Required for Producing Intracellular ATP in Xag**

Phosphoglycerate kinase is involved in energy-requiring activities, such as EPS biosynthesis and cell motility; therefore, we determine whether Pgk affects intracellular energy production in Xag. Our results showed that intracellular ATP of NΔpgk was 83.6 and 72.8% of the wild-type level when cultured for 12 and 24 h, respectively, in NY medium supplemented with 2% glucose (Figure 6). The ATP levels in CNΔpgk were completely restored to the wild-type level at all incubation times, indicating that pgk is required for producing intracellular ATP in Xag. Interestingly, by-path complementation of clp in NΔpgk did not increase or maintain intracellular ATP levels of NΔpgk; instead, it slightly decreased the ATP levels (Figure 6). Overall, these results reveal that Pgk is required for the production of ATP in Xag.

**Pgk Is Regulated by the HrpG/HrpX Cascade, but Not by DSF Signals or the Global Regulator Clp in Xag**

We determined whether the mRNA level of pgk is regulated by the DSF signals, low-oxygen signals, and the HrpG/HrpX cascade in Xag. Our results showed that the expression of pgk in the deletion mutants of rpfG, rpfS, rpfR, ravS, and ravR was significantly lower than that in the wild-type, but the deletion mutants of rpfF, rpfB, rpfC, and clp had similar expression levels as the wild-type strain, implying that the expression of pgk is not regulated by DSF signals or the global regulator Clp in Xag (Figure 7A). Compared with the wild-type, the expression of pgk was reduced in the deletion mutants of hrcC, hrcB, thr, xapL, zur, and rsmA, indicating that pgk is positively regulated by the HrpG/HrpX cascade (Figure 7B).

**Pgk Is Not Involved in the Production of Exoenzymes and in H₂O₂ Resistance of Xag**

Because pgk was positively regulated by Zur and RsmA, which are involved in regulating detoxification and exoenzymes production in Xanthomonas spp. (Büttnner and Bonas, 2010), we examined the effect of Pgk in the exoenzymes production and in H₂O₂ resistance in Xag. We found that the deletion of pgk in Xag did not affect the production of exoenzymes, such as protease, α-amylase, carboxymethylcellulase, and endo-β-mannanase (Supplementary Figure S4). We have previously demonstrated that either deletion or over-expression of clp reduces the protease activity, and Clp positively regulates synthesis of α-amylase, endo-β-mannanase, and carboxymethylcellulase in Xag (Guo et al., 2019). Therefore, the halos produced by NΔpgk with by-path complementation clp looks smaller in protease assay and larger in α-amylase, carboxymethylcellulase, and endo-β-mannanase assays when compared with NΔpgk (Supplementary Figure S4). In addition, the deletion of pgk in Xag did not render more sensitive to H₂O₂ (Supplementary Figure S5). These results indicate that Pgk is not involved in the production of exoenzymes or in H₂O₂ resistance in Xag.

**Pgk Contributes to Xag Virulence and Growth in Host Soybean**

We determined whether Pgk plays a vital role in the virulence of Xag in soybean. Our findings indicated that NΔpgk exhibited significantly reduced virulence in soybean relative to the wild-type (Figure 8A), and CNΔpgk had similar virulence as the wild-type. Furthermore, NΔpgk(clp) showed increased virulence compared with NΔpgk, but less virulence compared with CNΔpgk. The T3SS deletion mutant NΔhrcC completely lost virulence on soybean (Figure 8A).

To determine whether the impaired virulence of NΔpgk is associated with decreased bacterial growth, we explored the growth of bacterial cells infiltrated into soybean leaves. The colonies of NΔpgk were significantly fewer than that of the wild-type strain at each of time points (P < 0.01). The growth of NΔpgk could be fully restored by pgk in...
trans, whereas \( \Delta hrcC \) did not show increased growth in inoculated soybean tissue (Figure 8B). In addition, \( \Delta pgk(clp) \) showed increased growth in soybean leaves compared with \( \Delta pgk \), but was less than that of \( CN\Delta pgk \) (Figure 8B). Taken together, these results indicate that \( pgk \) is required for virulence and growth of \( Xag \), and by-path complementation of \( clp \) is beneficial to the virulence of \( \Delta pgk \) in host soybean.
Phosphoglycerate kinase is involved in cell motility of Xag independent of Clp. (A) Cell motility of NΔpgk, CNΔpgk, and NΔpgk(clp) was detected on NY plates or NY plates supplemented with 0.5% glucose and photographed after 1–2 days of incubation at 28°C. (B) Swimming diameter of NΔpgk, CNΔpgk, and NΔpgk(clp) on plates was measured. (C) The transcriptional levels of the flagellar-associated genes in NΔpgk, CNΔpgk, and NΔpgk(clp) were examined by qRT-PCR. Data are means ± SD from three replicates. The different letters above the horizontal columns represent significant differences at \( P < 0.01 \).
**DISCUSSION**

In this study, we found that Pgk plays an important role in the catabolism of glucose and other simple sugars in Xag. NΔpgk showed impaired growth when glucose, galactose, fructose, mannose, or sucrose was the sole carbon source (Figure 2). However, NΔpgk still showed certain level of growth in medium with these individual sugars as the sole carbon source, suggesting that Xag may employ ED and PPP, but not glycolysis, to utilize these sugars. This is consistent with the findings from previous studies conducted in the parenchyma pathogen Xoc (Guo et al., 2015), as well as the vascular pathogens X. campestris pv. campestris (Xcc) (Lu et al., 2009) and X. oryzae pv. oryzae (Xoo) (Kim et al., 2010), which lack phosphofructokinase activity required for glycolysis. In addition, NΔpgk shows no growth in medium with pyruvate as the sole carbon source (Figure 2), implying that inactivation of Pgk probably results in a severing of the gluconeogenic pathway. Similarly, this has also been observed in Xoc (Guo et al., 2017) and Xcc (Tang et al., 2005), in which the functional gluconeogenic pathway is necessary for the acquisition of pyruvate. Therefore, we deduce that Xag, probably similar to Xoc and Xcc, possesses an identical carbon metabolic pathway, using ED in conjunction with TCA to catabolize glucose. Interestingly, introduction of the recombinant plasmid pCclp into NΔpgk results in further impairment of the utilization of carbohydrates. This is consistent with results from a previous study on the overexpression of clp in Xag, which results in reduced utilizations of carbohydrates (Guo et al., 2019). Therefore, we speculate that overexpression or by-path complementation of clp may directly or indirectly inhibit the activity of a key metabolic enzyme from carbon metabolic pathways, leading to the limited acquisition of carbohydrates in Xag.

Our data showed that deletion of pgk in Xag resulted in significantly reduced EPS production (Figures 4A,B). This was further supported by the qRT-PCR results that showed that the expression of gum genes was significantly reduced in NΔpgk (Figure 4C). We also found that a mutation in pgk in Xag resulted in significantly reduced cell motility (Figure 5), which is similar to pgi (encoding glucose-6-phosphate isomerase) and zwf (encoding glucose-6-phosphate dehydrogenase) of Xoc (Guo et al., 2015, 2017). However, the decreased motility was not correlated with the expression of fliA, flhF, rpoN2, fleN, and fleQ (Figure 5C), which are required for cell motility (Tian et al., 2015). Pgk is involved in the reversible conversions between glycerate-1.3-2P and glycerate-3P in carbon metabolic pathways. Thus it is indicated that Pgk has a dual function in energy generation or consumption. Pgk is the producer of ATP in glycolytic pathway, but it is an ATP consumer in gluconeogenic pathway. In order to explore the possible reasons for the decrease of EPS synthesis and cell motility in NΔpgk, we investigated the intracellular ATP level, which provides energy to drive these activities (Chao et al., 2008). We found that mutation in pgk led to a reduced intracellular ATP level (Figure 6), which may result from the reduced acquisition of carbohydrates. Thus, we speculate that the reduced intracellular ATP level in NΔpgk may be one of the major causes that negatively affect EPS production and cell motility in this mutant strain.

Previous studies have shown that Clp directly or indirectly regulates diverse biological processes, including EPS production,
FIGURE 8 | Phosphoglycerate kinase is essential for the growth and virulence of Xag in soybean. (A) Bacterial pustules caused by Xag strains on inoculated leaves of soybean seedlings. Photographs were taken 15 days post-inoculation. (B) Bacterial growth in inoculated leaves of soybean. Bacteria were recovered from the inoculated leaves every day for 7 days post-inoculation and homogenized in sterile water. The homogenates were diluted and plated on NY plates with appropriate antibiotics. Bacterial CFU was counted after incubation at 28°C for 3 days. Data are means ± SD from three repeats.

FIGURE 9 | Working model to depict the association between Pgk and Clp in biological processes in Xag. Both pgk and clp, positively regulated by the HrpG/HrpX cascade, are involved in carbohydrate utilization, EPS biosynthesis, and cell motility of Xag. However, pgk is not regulated by either DSF signals or Clp, and is involved in the above biological properties independent of Clp.
cell motility, the TCA cycle, and the synthesis of extracellular enzymes (He et al., 2007; Guan et al., 2009). Recently, we also found that Clp functions as both an activator and/or a repressor in multiple biological processes, such as EPS production, cell motility, carbohydrate utilization, and extracellular enzyme activities (Guo et al., 2019). Thus, we further investigated the association between Pgk and Clp in these biological processes. However, by-path complementation of clp did not restore of the impaired properties caused by NΔpgk. Although pgk contains a putative Clp binding motif AGGCA-N6-TCACA in the promoter region (He et al., 2007), the transcriptional levels of pgk in the mutants of rpfF, and clp are equivalent to that in the wild-type (Figure 7A), indicating that pgk is not regulated by DSF signals/Clp regulon in Xag. Thus, we speculate that Pgk and Clp likely participate in diverse biological processes, such as carbohydrate utilization, cell motility, and EPS production, via different pathways.

A previous study has shown that the genes encoding catalytic enzymes in carbohydrate metabolic pathways could be regulated by the Hrp system in Xoc (Guo et al., 2017). Here, we found that pgk is also positively regulated by the HrpG/HrpX cascade and its upstream regulators RsmA and Zur (Figure 7B). Previous studies have reported that RsmA regulated EPS biosynthesis and cell motility, and Zur regulated EPS biosynthesis in Xanthomonas spp. (Chao et al., 2008; Huang et al., 2009; Zhu et al., 2011). Further studies should be conducted to investigate whether RsmA and Zur regulate EPS biosynthesis and cell motility partially through Pgk, or if both RsmA and Zur share the same signaling pathway as Pgk.

Our results showed that Pgk is essential for maintaining both virulence and growth of Xag in soybean (Figure 8). Attenuation in the virulence and growth of NΔpgk in host plant may have resulted, at least partially, from the combined effects of the compromised utilization of carbohydrates, the impaired production of EPS, and limited cell motility (Huang et al., 2009; Lu et al., 2009; Guo et al., 2019). Nutrient utilization is indispensable for pathogen to grow and colonize within host cells (Mellgren et al., 2009). Therefore, the reduced ability of NΔpgk to utilize carbohydrates in soybean reduces its growth and aggressiveness. In addition, previous studies have suggested that carbohydrates could induce metabolic changes, the secretion of extracellular enzymes, and the expression of virulence-related genes (Watt et al., 2009), which are crucial for the virulence of Xanthomonas spp. EPS can enhance susceptibility of host by repressing defense responses such as callose deposition (Yun et al., 2006), mask bacterium to prevent recognition by host (Lu et al., 2009), and contribute to biofilm formation (Dow et al., 2003), which play an important role during pathogen infection in Xanthomonas spp. Cell motility allows bacteria to obtain sufficiently nutritional sources, avoid unfavorable environments and disperse effectively, and seems to be required for the pathogenicity of the parenchyma pathogen Xanthomonas spp. (Malamud et al., 2011). Interestingly, by-path complementation of clp contributes to the virulence of NΔpgk to certain extent. The most likely reason is that significantly increased extracellular enzyme activity probably promotes the infection and colonization of pathogen in host soybean. Plant cell walls act as the first barrier of defense against bacterial invasion. Cell wall-degrading enzymes may facilitate pathogen invasion into host cells by digesting cell walls, thus contribute to disease development (Tayi et al., 2016).

The results from this study, together with the results from our previous study (Guo et al., 2019), advance our understanding of the biological properties of Pgk, an enzyme which reversibly catalyzes the conversions between glycerate-1.3-2P and glycerate-3P, and enable us to propose a working model to depict the functional roles of Pgk and Clp, and understand how their gene expression is regulated in Xag. In this model (Figure 9), both pgk and clp are positively regulated by the HrpG/HrpX cascade, which transduces environmental signals. In addition, clp is also positively regulated by DSF signals. However, pgk is not regulated by either DSF signals or Clp. Even though Clp and Pgk contribute positively to carbohydrate utilization, EPS biosynthesis, and cell motility, they are not functionally interconnected. It is likely that Pgk contributes to carbohydrate utilization, EPS biosynthesis, and cell mobility through generating metabolic carbon products and/or altering the intracellular ATP levels either directly or indirectly by its enzymatic activities. It seems that the function of Pgk in these processes is totally independent of Clp, the global regulator in Xanthomonas spp.

DATA AVAILABILITY STATEMENT
All datasets generated for this study are included in the article/Supplementary Material.

AUTHOR CONTRIBUTIONS
WG, G-YC, and J-ZL conceived the study. WG, JG, H-JW, R-YS, C-YG, and S-HG performed the experiments. WG and J-ZL analyzed the data. WG wrote the manuscript. J-ZL and G-YC revised the manuscript.

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SUPPLEMENTARY MATERIAL
The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.00091/full#supplementary-material
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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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