The Glaesserella parasuis phosphoglucomutase is partially required for lipooligosaccharide synthesis

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Abstract
Lipooligosaccharides (LOSs) are virulence determinants of Glaesserella parasuis, a pathogen of the respiratory tract of pigs. We previously reported that disruption of the galU or galE gene in G. parasuis results in increased sensitivity to porcine serum, indicating that the galactose catabolism pathway is required for polysaccharide formation in G. parasuis. Here, we evaluated the role of the HAPS_0849 gene in LOS synthesis. The G. parasuis SC096 HAPS_0849 mutant produced a highly truncated LOS molecule, although a small fraction of intact LOS was still observed, and this mutant was found to be more sensitive to serum than the parental strain. HAPS_0849 was overexpressed and purified for biochemical assays, and this protein exhibited phosphoglucomutase (PGM) activity. Heterologous expression of a pgm gene from Escherichia coli in the HAPS_0849 mutant led to restoration of the wild-type LOS glycoform, further demonstrating the PGM function of HAPS_0849 in G. parasuis. The autoagglutination and biofilm formation ability of this strain were also investigated. Disruption of HAPS_0849 led to an increased tendency to autoagglutinate and form more biofilms, and these enhanced phenotypes were observed in the absence of glucose. In addition, LOSs from HAPS_0849, galU and lgtB mutants had similar truncated glycoforms, while LOSs from the galE and lex-1 mutants exhibited another type of defective LOS pattern. These findings imply that HAPS_0849 may function upstream of GalU in the generation of glucose 1-phosphate. In conclusion, our results preliminarily described the functions of HAPS_0849 in G. parasuis, and this gene was partially required for LOS synthesis.

Keywords: Glaesserella parasuis, lipooligosaccharide, phosphoglucomutase, biofilm

Introduction
Glaesserella parasuis is a pathogen of the upper respiratory tract of conventional swine herds and the aetiological factor of Glässer's disease, causing fibrinous polyserositis, arthritis, and meningitis. G. parasuis has emerged as one of the major causes of high mortality rates in piglets, resulting in significant economic losses to the swine industry worldwide [1, 2]. The complement system is part of the innate immune system and is a first line of defence. The cascade of reactions that follows complement activation can lead to the formation of the membrane attack complex (MAC), resulting in lysis of the pathogen [3]. The complement susceptibility of G. parasuis strains of different clinical origins is highly variable. In general, isolates from the nares of healthy piglets are sensitive to the bactericidal effect of the complement system, while strains from systemic diseases of swine tend to be resistant to serum [4]. In a previous study, the capD gene, encoding a capsular polysaccharide biosynthesis protein, was identified as a virulence-associated factor, indicating that surface polysaccharides may participate in virulence, particularly in the context of serum resistance in G. parasuis [5].

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Bacterial polysaccharides (PCs), including capsular polysaccharides (CPSs) and lipooligosaccharides (LOSs), are widespread structures found on the cell surfaces of a broad range of Haemophilus species and have been confirmed as major complement resistance factors in these bacteria [6–10]. Capsules were previously observed in some G. parasuis strains by precipitation with hexadecyl trimethylammonium bromide (CTAB), and the presence of acidic polysaccharides in some types of capsules was observed. However, capsules from other strains did not coprecipitate with CTAB, suggesting that the composition of these capsules may be quite different or that CPS is not synthesized by these strains [11]. This finding may be attributed to the genetic diversity of the capsular bio-synthetic locus in strains of different serotypes [12]. Furthermore, three heptosyltransferase genes (opsX, rfaF, and waaQ) are required for lipooligosaccharide (LOS) synthesis. Disruption of these genes resulted in serum-sensitive mutants, indicating that LOSs are also needed for virulence of G. parasuis [13].

The biosynthesis of surface polysaccharides is closely related to carbohydrate metabolism in pathogens [14–16]. In our previous study, two galactose metabolism-relevant genes, denoted galU and galE, were found to affect virulence and biofilm formation in G. parasuis SC096 [17]. The metabolism of galactose may affect the synthesis of CPS or LOS, as galU and galE mutants are notably more sensitive to killing by porcine serum than their parent strain. As shown in Figure 1, exogenous glucose or amino acids can be converted to glucose 1-phosphate (G1P) by glycolysis then converted to uridine diphosphate glucose (UDP-glucose) by UDP-glucose pyrophosphorylase (GalU) and further converted to UDP-galactose by UDP-glucose-4-epimerase (GalE). These monosaccharide derivatives can then be utilized for the synthesis of various polysaccharides by glycosyltransferases, such as lgtB and lex-I, as described in our previous study [18]. To date, the genome sequences of many G. parasuis strains have been determined [19–22], and several virulence factors involved in the evasion of immune responses have been identified [5, 13, 17, 23–25]. However, the correlation between UDP-sugars biosynthesis and virulence has yet to be further investigated. The gene HAPS_0849 (NCBI accession no. ACL32480.1) in the sequenced strain SH0165 is annotated as encoding phospho-sugar mutase. This mutase may be an upper-pathway enzyme involved in the generation of G1P, which is further used for LOS synthesis by GalU as described above (Figure 1). The phosphohexomutase family includes several homologous enzymes that catalyse the reversible conversion of 1-phospho-sugar substrates to 6-phospho-sugar substrates. The proteins in this superfamily play important roles in carbon source metabolism in bacteria. At least three related proteins constitute the majority of this superfamily: the highly specific phosphoglucomutase (PGM) protein, which uses only glucose as a substrate, the mannose specific phosphomannomutase (PMM) protein, and the less specific PMM/PGM protein, a bifunctional protein that can use either mannose or glucose [26]. HAPS_0849 is homologous to the gene pgmB, which is predicted to encode a PGM and is involved in LOS synthesis in Haemophilus influenzae [27]. However, the biochemical functions of these genes in Haemophilus species are still unclear. Importantly, the correlation between PGM and the source of G1P mentioned above, as well as the roles of PGM in G. parasuis, remain poorly understood. Surface polysaccharides of G. parasuis are important virulence factors and key antigens for vaccine development. The pathway pgm-galU-galE may be a key node for these polysaccharides biosynthesis based on our studies although it is yet to be investigated further. How these polysaccharides synthesis affected by metabolism, and how to improve the yield of these key antigens? Understanding of G. parasuis pgm may provide some hints to answer these questions.

In the present study, we identified the gene HAPS_0849, encoding a PGM, as a virulence factor in G. parasuis. Phenotypes of its mutant affected by concentration of glucose are different from that of other reported LOS mutants in G. parasuis, and interestingly the HAPS_0849 gene is partially required for lipooligosaccharide synthesis. To our best knowledge, it may be the first report to show these findings in Pasteurellaceae species and even in other bacteria. We have attempted to explain the relevance between PGM and polysaccharide synthesis in G. parasuis and pinpoint the molecular basis of this link.

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**Figure 1** Schematic representation of UDP-sugars biosynthesis in G. parasuis. The genes involved in polysaccharide synthesis with experimental evidence are marked in blue. The HAPS_0849 gene, annotated as a phosphoglucomutase, is marked as red.
Materials and methods

Bacterial strains, plasmids, and growth conditions

The bacterial strains and plasmids used in this study are listed in Additional files 1 and 2. *Escherichia coli* strains were routinely grown in Luria–Bertani (LB) broth or on LB agar plates at 37 °C. *G. parasuis* strains were cultured on trypticase soy agar (TSA) or trypticase soy broth (TSB) with or without dextrose (BD Biosciences, USA) and supplemented with 0.005% nicotinamide adenine dinucleotide (NAD) (Sigma Aldrich, USA) and 5% inactivated bovine serum at 37 °C in 5% CO₂ as previously described [17]. When required, the media were supplemented with kanamycin (30 μg/mL) or gentamicin (30 μg/mL).

Molecular biology methods

Plasmid isolation was performed using the QIAprep Spin Miniprep Kit (Qiagen, Germany). Restriction enzymes were purchased from New England BioLabs Inc. T4 DNA ligase, Taq polymerase and high-fidelity DNA polymerase were purchased from Takara Bio Inc., Japan. Restriction enzyme digestion, ligation, and transformation of *E. coli* were performed using standard methods. Genomic DNA was extracted with the E.Z.N.A. Bacterial Genomic DNA Kit (Omega Bio-Tek, USA). PCRs were used to produce DNA fragments and evaluate mutants.

Construction of plasmids and strains

The primers used in this study are listed in Additional file 3. Mutants were constructed as previously described [17] with some modification. To mutate the *G. parasuis* *HAPS_0849* gene, regions of homology flanking the *HAPS_0849* locus were amplified by PCR using SC096 genomic DNA as a template. The upstream region was amplified using primers P1 and P2, while the downstream fragment was amplified using primers P3 and P4. The KanR gene was amplified from the plasmid template pK18mobsacB with primers P11 and P12, and these three fragments were joined by overlap PCR with the primer pair P1 and P4. This PCR product was then cloned into pMD19-T (Takara Bio Inc., Japan) to generate pSF251. The constructed plasmid was then used for natural transformation, and the *HAPS_0849* mutant was generated with the method described previously [23]. Briefly, bacteria were cultivated overnight at 37 °C on TSA plates and then washed and resuspended in TSB supplemented with newborn calf serum and NAD at 5 × 10⁵ CFU/mL. A 20-μL bacterial suspension was pipetted onto TSA supplemented with inactivated serum and coenzyme and spread onto a small area. One microgram of the plasmid DNA was then added and mixed, followed by cultivation for 5 h at 37 °C. Cultures were scraped off and plated on TSA with 30 μg/mL kanamycin and then incubated at 37 °C for 2 days. Positive clones were evaluated by PCR and sequencing. The *wclP* and *wcaJ* mutant strains were constructed by a similar method with the primers P5-P8 and P33-P36 listed in Additional file 3.

Complemented strains were constructed using the method published previously [28] with some modifications. Intact *HAPS_0849* was amplified with primers P13 and P14. This PCR fragment was digested with Sall and Sphi, subjected to agarose gel electrophoresis followed by purification with the QIAquick Gel Extraction Kit (Qiagen, Germany), and ligated into pSF116, a complementation vector for inserting target genes between *ompP5* and *msrB*, to generate pSF254. This recombinant plasmid was transformed into the *HAPS_0849* mutant by natural transformation, and selection of the complemented *G. parasuis* *ΔHAPS_0849* strain (SF415) was accomplished using TSA containing gentamycin. Transformants were confirmed by PCR and sequencing using primers P5 and P10. The *ΔwclP* and *ΔwcaJ* complemented strains (SF416 and SF417, respectively) were constructed using a similar method as that described above with the corresponding primers (Additional file 3).

The *ΔHAPS_0849* strain expressing *E. coli* *pgm* was constructed as follows: the *pgm* gene fragment was amplified from *E. coli* MG1655 genomic DNA by PCR with primers P19 and P20. After purification and digestion with Sall and Sphi, *Ecpgm* was ligated into pSF116 to create pSF257. This plasmid was then transformed into the *ΔHAPS_0849* strain by natural transformation and selected using gentamicin (Gm). Positive clones were confirmed by PCR amplification and sequencing using primers P5 and P10.

To construct the *G. parasuis* strain overexpressing *HAPS_0849*, complete open reading frame of *HAPS_0849* was amplified by PCR using the primer pair P39 and P40. After gel extraction, the PCR product and pET28(a) vector were digested with NdeI and HindIII, gel purified, and ligated together to corresponding sites using DNA ligase, generating the expression plasmid pSF258. The correct recombination was confirmed by PCR and sequencing.

Overexpression and purification of *G. parasuis* *HAPS_0849*

pET28–*HAPS_0849* was transformed into *E. coli* BL21 (DE3) for overexpression. A single colony was selected and cultured in a flask overnight and then used to inoculate 200 mL of LB broth supplemented with kanamycin at a concentration of 50 μg/mL. The cells were cultivated at 37 °C, and then isopropyl-β-d-thiogalactoside (IPTG) was added at a concentration of 1 mM when the absorbance OD₆₀₀ reached 0.8. The induced cells were incubated for another 4 h and then centrifuged to obtain a bacterial pellet. Next, the cells were resuspended in 1/5 volume
of lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole, pH 8.0) and lysed by ultrasonic treatment for 20 min. Supernatants were collected after centrifugation at 13,400 × g for 10 min, and then the lysate was filtered with a 0.22 µm Millipore polyethersulfone filter. Nickel-nitrilotriacetic acid (Ni-NTA) matrices (GE Healthcare) were used for protein purification. The Ni-NTA column was preequilibrated with ten volumes of lysis buffer, and then the samples were loaded onto the column by gravity. After washing with ten volumes of washing buffer (50 mM NaH₂PO₄, 300 mM NaCl, 20 mM imidazole, pH 8.0), target proteins were then eluted with 2 mL of elution buffer (50 mM NaH₂PO₄, 300 mM NaCl, 500 mM imidazole, pH 8.0) in 4 fractions. The purified proteins were analysed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The fractions with pure protein were pooled and dialyzed against dialysis buffer with dialysis buffer for further enzyme activity analysis.

Phosphoglcomutase activity assay
The activity of HAPS 0849 was confirmed with a Glucose-6-Phosphate Assay Kit (Sigma, MAK014) according to the manufacturer’s manual, with some modifications. Briefly, 10 µL of 1 mM G1P was added to a 96-well plate, and then G6P assay buffer was added to each well to bring the volume to 50 µL. Another 50-µL reaction mix containing 45 µL of G6P Assay Buffer, 2 µL of G6P Enzyme Mix, 2 µL of G6P Substrate Mix and 1 µL of protein sample or phosphate buffered saline (PBS) as a negative control was prepared. Next, 50 µL of the reaction mix was added to 50 µL of the 1 mM G1P solution described above and mixed well by pipetting. The reaction was incubated for 30 min at room temperature, and then the absorbance was measured at 450 nm (A450). To eliminate the effect of background NADH or NADPH, a blank sample was prepared for each sample by omitting the glucose-6-phosphate enzyme mix. The blank readings were subtracted from the sample readings. Activity was calculated as U (units) = (Aₜₐₐ − Aₐₓ)/(T₁ − T₀) × df (where Aₓ, A₄₅₀ of sample after 30 min; Aₜ, A₄₅₀ of sample without G6P enzyme mix as a blank; T₁, time reaction was stopped; T₀, time reaction began; df, dilution factor of protein). Phosphoglcomutase from rabbit muscle (Sigma Aldrich, USA) was used as positive control.

Autoagglutination assay
The ability of *G. parasuis* strains to autoagglutinate was tested by a modified method as previously reported [17]. Briefly, *G. parasuis* strains were cultured overnight in 100 mL of TSB broth with or without glucose and supplemented with 0.005% NAD and 5% inactivated serum and diluted to an optical density at 600 nm of one. The bacterial cultures were then transferred to 15 mL tubes and allowed to remain static at room temperature. The turbidity of the culture was determined every 60 min for 12 h.

Biofilm formation assay
The biofilm formation test was performed as previously reported with some modifications [29, 30]. Briefly, 20 µL of overnight culture of the wild-type strain and its mutants were inoculated into 2 mL of fresh TSB broth containing NAD and inactivated serum with or without glucose in 6-well plates, and then incubated for 24 h or 72 h at 37 °C. Supernatants were removed by pipetting; then, biofilms were rinsed with sterile distilled water and stained with 0.1% crystal violet for 30 min. Afterwards, excess staining was gently washed off with dH₂O, and the biofilms were dried. Bound crystal violet was dissolved in 1 mL of 33% (v/v) acetic acid, and 200 µL of solution was transferred to a 96-well microtiter plate. Then, the optical density was measured at 600 nm. All experiments were performed independently three times.

Serum sensitivity assay
The serum bactericidal assay was carried out as previously described with 50% porcine serum, which was also obtained from a previous study [17]. Serum inactivated at 56 °C for 30 min was used as a control in all experiments. The wild-type strain and mutants were cultured to mid-logarithmic phase, and then, 100 µL of porcine serum or inactivated serum was added to 100 µL of a bacterial dilution (10⁸ CFU), followed by incubation at 37 °C for 1 h. The bacterial cells were then serially diluted tenfold and plated on TSA plates supplemented with serum and NAD. The plates were cultivated at 37 °C in an atmosphere containing 5% CO₂ for 48 h. The survival percentage was determined according to the ratio of bacterial colonies in natural serum to those in heat-inactivated serum. All strains were tested in at least three independent experiments.

Preparation of LOSs and electrophoresis
Bacterial polysaccharides were prepared from *G. parasuis* strains as described previously [18]. Bacterial cells were grown on TSA supplemented with NAD and 5% newborn calf serum (NBS) and washed with PBS, and LOSs were extracted with a concentration of 50% phenol at 70 °C for 15 min. The resulting aqueous phase was dialyzed against distilled water until phenol was removed (molecular weight cutoff, 1000 Da). The samples were separated by 15% SDS-PAGE, and the gels were visualized using silver staining.
**Statistical analysis**

All experiments were performed at least three times, and the standard deviations were calculated with GraphPad Prism. Student’s t-test was used to evaluate the significance of differences. A P-value < 0.05 was considered statistically significant.

**Results**

The *HAPS_0849* mutant has defects in LOS synthesis and is sensitive to serum

To investigate the function of *HAPS_0849*, a kanamycin resistance cassette with flanked regions of this gene was used to disrupt *HAPS_0849* on the chromosome of strain SC096, and the generation of the complemented strain is described in “Materials and methods” section (Additional file 4). Our previous studies have shown that the heptosyltransferase genes *opsX*, *rfaF*, and *waaQ* are required for the transfer of heptoses I, II, and III, respectively, in *G. parasuis* [13], and these heptoses are derived from UDP-glucose, which is generated through the *galUE* pathway. Therefore, we sought to determine whether *HAPS_0849* was involved in lipoooligosaccharide synthesis and participated in the generation of UDP-glucose (Figure 1). The LOSs from the *HAPS_0849* mutant, along with the wild-type strain SC096, were visualized by SDS-PAGE and silver staining. Figure 2A shows that SC096 expressed a single LOS band (lane 1), and Δ*HAPS_0849* produced two types of LOS glycoforms (lane 2) when these strains were cultured in TSB with 0.25% glucose. The principal LOS glycoform of Δ*HAPS_0849* migrated faster than the wild-type LOS, which suggests that the *HAPS_0849* mutant synthesized a highly truncated LOS molecule. Notably, Δ*HAPS_0849* also partially produced intact LOS, with a banding pattern corresponding to that of the wild-type LOS. However, the proportion of complete LOS was obviously lower when this mutant was cultured in TSB without glucose (Figure 2B, lane 2). In addition, LOS glycoforms from the complemented *HAPS_0849* mutant displayed a migration profile that was consistent with the migration profile of LOS from the wild-type strain (lane 3 in Figures 2A, B).

LOSs and outer membrane proteins have been proposed to play a role in serum resistance in *G. parasuis* [13, 23]. To investigate whether *HAPS_0849* also contributes to serum resistance, the sensitivity of Δ*HAPS_0849* to 50% swine serum was investigated. The survival rates of wild-type strain SC096, *HAPS_0849* mutant and complemented strain were 73.16 ± 4.26%, 0.052 ± 0.046% and 76.68 ± 4.06% respectively. The bactericidal activity of swine serum with the *HAPS_0849* mutant was significantly greater than that of the parent strain *G. parasuis* SC096 (*P* < 0.01), whereas the serum resistance phenotype was restored in the complemented strain (Figure 3). It should be noted that there was negligible variance in the doubling times between Δ*HAPS_0849* and the wild-type strain SC096 (Additional file 5). These results suggest that *HAPS_0849*...
was partially required for LOS synthesis and that the HAPS_0849 mutant was sensitive to the bactericidal effects of porcine serum.

**HAPS_0849 encodes a phosphoglucomutase**

In order to confirm the biochemical functions of HAPS_0849, we constructed a pET28-HAPS_0849 plasmid and transformed it into *E. coli* strain BL21 (DE3) for overexpression. After IPTG induction and ultrasonication, the concentration of target protein in the soluble fraction was high. Afterwards, we purified the proteins from the supernatant solution directly with Ni-NTA resin. After affinity chromatography, the HAPS_0849 protein was diluted to the appropriate concentration, analysed by SDS-PAGE (Figure 4A) and further confirmed by matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) analysis (data not shown). For enzyme activity analysis, a modified phosphoglucomutase assay was performed as described in “Materials and methods” section. The purified protein had a significant ability (P < 0.05) to convert glucose 1-phosphate to glucose 6-phosphate generation compared to the negative control (Figure 4B), indicating that HAPS_0849 may have phosphoglucomutase activity.

The *E. coli pgm* gene (*Ecpgm*) encodes phosphoglucomutase, which is specifically essential for the conversion of glucose 6-phosphate to glucose 1-phosphate [31]. To further assess whether *G. parasuis* HAPS_0849 is a phosphoglucomutase, we constructed an Ecpgm-expressing strain derived from the HAPS_0849 mutant (Additional

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**Figure 3** Survival of the HAPS_0849 mutant treated with 50% porcine serum. The ΔHAPS_0849 mutant strain showed significantly increased sensitivity to 50% porcine serum compared to the wild-type strain SC096 (*P* < 0.01). The serum resistance phenotype was restored in the complemented strain. Error bars are standard errors of the means (SEM) from three independent experiments.

**Figure 4** Analysis of HAPS_0849 by activity assay and gene complementation with *E. coli pgm*. **A** Expression and purification of HAPS_0849 in E. coli BL21 (DE3). Lane M, protein molecular marker; lane 1, purified and diluted HAPS_0849. **B** PGM activity assay described in “Materials and methods” section. G1P was used as the substrate; the reaction started only with 6-phosphoglucose dehydrogenase (G6PDH) was set as the negative control (NC), OcPGM was from rabbit muscle as positive control. In comparison with the NC, HAPS_0849 exhibited significant PGM activities in the enzyme-linked reaction (*P* < 0.05). Bars represent the mean ± standard error of three independent experiments. **C** LOS profiles of the *G. parasuis* SC096, ΔHAPS_0849 and Ecpgm-expressing strains. Lane M, protein molecular marker; lane 1, wild-type; lane 2, ΔHAPS_0849 mutant; lane 3, ΔHAPS_0849-ompP5::Ecpgm.
file 6). LOSs were extracted and analysed by electrophoresis; as shown in Figure 4C, a principal LOS glycoform from the pgm-expressing strain was disrupted in HAPS_0849 (lane 3), with an apparent molecular weight equivalent to that of the major LOS profile expressed by the wild-type strain (lane 1). Moreover, a small LOS glycoform was mainly observed in ΔHAPS_0849, as predicted (lane 2). Complementation of the HAPS_0849 mutant with the Ecpgm gene also implied that HAPS_0849 may mainly function as a phosphoglucomutase in G. parasuis.

Disruption of HAPS_0849 enhances autoagglutination and biofilm formation

The serum-sensitive phenotype and LOS truncation of ΔHAPS_0849 indicate that HAPS_0849 may be a virulence gene in G. parasuis. To further investigate the role of this gene, autoagglutination and biofilm formation assays were carried out as previously described. The aggregation results showed no obvious difference between SC096 and ΔHAPS_0849 cultured in TSB with 0.25% glucose in the first 10 h; however, slight precipitation was observed after incubation was prolonged for 2 h (Figure 5A, D). The decrease in the optical density of ΔHAPS_0849 dramatically accelerated when the strain was precultured in the same medium with a lower concentration (0.05%) of glucose (Figure 5B). Meanwhile, no apparent decrease in absorbance was observed in the wild-type and complemented strains. Furthermore, the aggregation of ΔHAPS_0849 cultured in a lower glucose concentration was more obvious than that cultured in a higher glucose concentration, as shown in Figure 5E. The absorbance of ΔHAPS_0849 at 600 nm drastically declined and reached its lowest value in 6 h when the strain was cultivated in TSB broth without glucose compared to the absorbance of SC096 (Figure 5C). The accumulation amount of ΔHAPS_0849 cells in this condition was similar to that in 0.05% glucose after 12 h of incubation, while the phenotype of the complement strain was consistent with that of the parent strain (Figure 5F). The results indicated that the HAPS_0849 mutant was inclined to autoagglutination and that the sedimentation rate was influenced by the concentration of glucose.

The biofilm formation abilities of the wild-type and ΔHAPS_0849 strains were assessed, and the results are depicted in Figure 6. After 24 h or 72 h, a significant gradual increase (P < 0.05) in biofilm biomass of ΔHAPS_0849 was observed compared to the biofilm formed by SC096 or the complemented strain in the absence of glucose (Figure 6A, B). The absorbance of ΔHAPS_0849 at 600 nm was close to 1.0 after 24 h and 2.0 after 72 h, while the absorbance values of the wild-type and complemented strains were less than 0.5 at both time points. The absorbance values of the stained biofilms of all strains

*Figure 5* Autoagglutination of *G. parasuis* SC096, ΔHAPS_0849 and the HAPS_0849 complementation strain. A Autoagglutination rates of the SC096, ΔHAPS_0849 and ΔHAPS_0849-comp strains cultured in TSB with 0.25% glucose. D A photograph was taken at the end of the assay, as shown in panel A, and the results were from a representative experiment. Lane 1, SC096; lane 2, ΔHAPS_0849 mutant; lane 3, complemented strain B, E Assays were performed under the same conditions corresponding to panels A and D except that strains were cultivated in TSB broth with 0.05% glucose. C, F Tests were carried out in the same conditions corresponding to panels A and D except that strains were cultured without glucose. With the decrease in glucose, the rates of autoagglutination increased gradually.
did not exceed 0.5 when the strains were incubated in the presence of 0.25% glucose at different time intervals (Figure 6C, D). Hence, this evidence further supported the influence of glucose on the ΔHAPS_0849 strain, and disruption of the HAPS_0849 gene may increase biofilm formation in the absence of glucose.

**HAPS_0849 may be an upper-pathway enzyme in uridine diphosphate galactose synthesis**

We presented evidence that HAPS_0849 is a phospho-glucomutase that is responsible for producing G1P from G6P. Moreover, autoagglutination and biofilm phenotypes of ΔHAPS_0849 indicated that HAPS_0849 may correlate with surface polysaccharides biosynthesis. Thus, given our previous findings, we investigated whether HAPS_0849 was an upper-pathway gene in the gallUE pathway, as mentioned in “Introduction” section and Figure 1. Several mutants involved in polysaccharide synthesis were selected for LOS pattern comparison to explain the functional relationship between these genes. Two gallUE pathway mutants of G. parasuis strain SC096, namely, ZY001 (gallU) and ZY005 (gallE), defective in serum resistance have previously been reported [17]. Analysis of the ZY001 and ZY005 LOSs by silver-stained PAGE revealed that loss of gallU or gallE alone in SC096 resulted in the truncation of the LOS core (Figure 7A). The LOS core from the gallU mutant strain (lane 2) was smaller than that isolated from the gallE mutant strain (lane 4). In general, the patterns of LOSs from the complemented strains were the same as those of the LOSs isolated from the parent strain SC096 (lanes 1, 3 and 5), except that the truncation of the LOS core was not completely eliminated in the ΔgallU-complemented strain ZY002 (ΔgallU-c) (lane 3), but the serum resistance phenotype was restored [17]. These findings suggested that gallU and gallE are required for LOS completeness, and disruption of gallU or gallE resulted in different LOS patterns.

Our previous data demonstrated that mutants of two additional glycosyltransferases, lgtB or lex-1, exhibited alterations in the LOS core [18]. To investigate the LOS patterns of these G. parasuis mutants, LOSs from the

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**Figure 6** Biofilm formation by SC096 and the HAPS_0849 mutant and complementation strains. A Quantification of biofilm production from 24-h or 72-h cultures in TSB broth without glucose. This graph indicated that disruption of HAPS_0849 significantly increased the ability of the strain to form biofilms (*P < 0.05), while the biofilm formation phenotype of the complemented strain was restored to that of the wild-type strain SC096. B A photograph was taken at the end of the assay after cultivation in TSB with 0.25% glucose for 24 h or 72 h. C, D Experiments were performed under the same conditions except that strains were cultured with 0.25% glucose. These results showed that no obvious variance in the tested strains was observed when they were cultivated with adequate glucose. Error bars represent the standard deviations of three independent experiments.
The mutant (lane 4) produced a band identical to that of the major glycoform produced by the wild-type strain (lane 1) based on SDS-PAGE. This result suggests that the ΔgalU mutant may have similar phenotypes as the HAPS_0849 mutant based on the known pathway. However, the observation that the LOS pattern of ΔgalU was not completely consistent with that of ΔHAPS_0849 was unexpected. Unlike the phenotypes observed for ΔgalU, glucose clearly affected autoagglutination and biofilm formation in ΔHAPS_0849 [17], we hypothesize that the HAPS_0849 mutant may have similar phenotypes as the ΔgalU mutant based on the observed LOS truncation of LOS in the HAPS_0849 mutant strain.

Discussion

PGM is responsible for the conversion of G6P to G1P and functions upstream of the galE pathway. This protein is important for the virulence of a number of bacterial pathogens, including Neisseria meningitidis [33], Brucella abortus [34], Bordetella bronchiseptica [35] and Salmonella enterica serovar Typhimurium [36] and is a critical enzyme connecting glycolysis with the synthesis of polysaccharides, such as CPS, LPS and EPS (exopolysaccharides). Based on our previous findings that galU and galE contribute to serum resistance and biofilm formation in G. parasuis [13, 18], we hypothesize that the HAPS_0849 mutant may have similar phenotypes as the ΔgalU mutant based on the known pathway. However, the observation that the LOS pattern of ΔHAPS_0849 was not completely consistent with that of ΔgalU and shared a partially identical band with the LOS of the parent strain was unexpected. Unlike the phenotypes observed for ΔgalU, glucose clearly affected autoagglutination and biofilm formation in the ΔHAPS_0849 strain. This finding pointed out the main difference between ΔgalU and the HAPS_0849 mutant. The UDP-glucose generated by GalU appeared to be the sole source for LOS synthesis.
because the LOS in ΔgalII was completely truncated. However, the substrate G1P converted by HAPS_0849 was not the only substrate according to our data provided here.

We highlight the importance of G1P because it is a central node involved in multiple carbon source pathways, including glycolysis, a central system of glucose metabolism. Because there are many phenotypes related to glucose, we suspect that there is another pathway to bypass the deficiency of G1P synthesis in the HAPS_0849 mutant. G1P can be derived from at least four routes based on Kyoto Encyclopedia of Genes and Genomes (KEGG) database analysis. As shown in Additional file 9, (1) G1P can be derived from endogenous glycogen that can be degraded by glyogen phosphorylase (GlgP); however, it should be noted that endogenous glycogen is derived from G1P, and glycogen synthesis can occur only when G1P is present. (2) G1P can be derived from the metabolism of galactose, catalysed by galactokinase (GalK) and galactose-1-phosphate uridylyl transferase (GalT); however, no phenotypic variation was observed in mutants lacking these enzymes (data not shown). (3) G1P can be derived from exogenous glycogen, such as dextrin, which can also be converted by GlgP. The metabolic fingerprint of SC096 shows that dextrin is an ideal carbon source for G. parasuis (unpublished observation), and dextrin has been proposed as a potential source for G1P supplementation. Finally, (4) G1P can be derived from G6P produced by HAPS_0849, which should be the principal source for G1P generation according to the data presented above.

Several studies have demonstrated that CPS and LOS are important virulence factors and confer serum resistance in G. parasuis [5, 12, 13, 37]. However, the correlation between these polysaccharides and carbon source metabolism still awaits experimental investigation. Our previous work reported that two glycosyltransferases annotated as lgtB and lex-1 are involved in the synthesis of LOS, and the absence of one of these genes can lead to a serum-sensitive phenotype [18]. Analysis of the LOSs of the mutants in this study showed that the LOS migration patterns of galE and lex-1 were consistent, while the LOSs produced by lgtB, HAPS_0849 and galU showed another glycoform. These results suggest that UDP-glucosyltransferase may serve as a substrate for lgtB, while lex-1 may use UDP-galactose as a donor for LOS synthesis, as the products of GalE and GalU are UDP-galactose and UDP-glucose, respectively. To date, only one study has performed structural analysis of the LOSs and CPSs of G. parasuis, and the results showed that multiple glucose and galactose residues do indeed exist in LOS molecules [32]. This finding may explain why the absence of galU or galE resulted in LOS truncation. In addition, these UDP sugars may also be involved in the synthesis of capsules because disruption of CPS genes reduced the resistance of G. parasuis to serum, and these genes are clearly not related to LOS expression. There are at least two types of polysaccharides that protect this bacterium from the effects of serum, and G1P could be a critical junction between glucose and UDP-sugars for these polysaccharides synthesis in G. parasuis.

In summary, we have identified HAPS_0849 as a phosphoglucomutase gene that is correlated with the biosynthesis of LOS in G. parasuis SC096. The HAPS_0849 mutant is not resistant to serum, and the complemented strain displayed a restored phenotype corresponding to that of the parent strain. Autoagglutination and biofilm formation were significantly increased in the ΔHAPS_0849 strain. Moreover, the LOS profile of the HAPS_0849 mutant was partially consistent with that of ΔgalU and HAPS_0849 may act upstream of galU in the generation of G1P. Altogether, our observations highlight the fact that PGM is a key node for LOS synthesis in G. parasuis.

**Supplementary information**

Supplementary information accompanies this paper at [https://doi.org/10.1186/s13567-020-00822-9](https://doi.org/10.1186/s13567-020-00822-9).

Additional file 1. Bacterial strains used in this study.
Additional file 2. Plasmids used in this study.
Additional file 3. Sequences of the PCR primers used in this study.
Additional file 4. Construction of in-frame deletion and complementation strains of the HAPS_0849 gene in G. parasuis (A and B). Schematic diagram of the HAPS_0849 mutant or complementation strain constructed in this study. Abbreviations: Kan’, kanamycin resistance gene; Gm’, gentamicin resistance gene; Up, upstream sequence of HAPS_0849; Dn, downstream sequence of HAPS_0849. (C) Locus structure of the HAPS_0849 mutant or complementation strain. Detection primers are shown as solid black arrows. (D) PCR analysis confirming the constructs. Lane M, DNA molecular marker; lanes 1–3, SC096, ΔHAPS_0849 and complementation strain with primers P21 and P22, as shown in this figure; lanes 4–6, the same strains with primers P23 and P24; lanes 7 and 8, ΔHAPS_0849 and its complementation strain with primers P9 and P10.

Additional file 5. Growth of SC096 and ΔHAPS_0849. Wild-type strain and ΔHAPS_0849 were cultured in TSB broth containing 5% inactivated bovine serum and 0.005% NAD and supplemented with (A) or without (B) 0.25% glucose. Error bars represent the standard deviations of three independent experiments.

Additional file 6. Construction of the E. coli pgm expression strain in the HAPS_0849 mutant. (A) Schematic diagram of the HAPS_0849 mutant or Ecpgm expression strain constructed in this study. (B) Locus structure of the HAPS_0849 mutant or Ecpgm expression strain. Detection primers are shown as solid black arrows. (C) PCR analysis confirming the constructs. Lane M, DNA molecular marker; lane 1, ΔHAPS_0849; and lane 2, ΔHAPS_0849ompP5:Ecpgm.

Additional file 7. LOS Structure of G. parasuis. Decayed LOS of G. parasuis strains ER-6P and Nagasaki [32]. GalN, galactosamine; Gal, galactose; GlcN, Glucosamine; Glc, glucose; Kdo, 3-deoxy-D-manno-octulosonic acid; Hep, heptose. Dotted lines indicate potential attached site of different monosaccharides for glycosyltransferase LgtB (red) and Lex-1 (blue).
Additional file 8. Construction and phenotype analysis of ΔwclP, ΔwcaJ and the corresponding complementation strains of G. parasuis. (A) PCR analysis verifying the ΔwclP constructs. Lane M, DNA molecular marker, lanes 1–3, SC096, ΔwclP and the complementation strain with primers P25 and P26, as shown in this figure part C; lanes 4–6, the same strains with primers P27 and P28; lanes 7 and 8, ΔwclP and its complementation strain with primers P9 and P10. (B) PCR analysis confirming the ΔwcaJ constructs. Lane M, DNA molecular marker, lanes 1–3, SC096, ΔwcaJ and the complementation strain with primers P29 and P30, as shown in this figure part C; lanes 4–6, the same strains with primers P31 and P32; lanes 7 and 8, ΔwcaJ and its complementation strain with primers P9 and P10. (C) Locus structure of wclP, wcaJ and the corresponding complementation strains. Detection primers are shown as black solid arrows. (D) Silver-stained SDS-PAGE gel of LOSs isolated from SC096 (lane 1), ΔwclP (lane 2) and ΔwcaJ (lane 3). (E) Survival of the wclP or wcaJ mutant treated with porcine serum. The ΔwclP or ΔwcaJ single-mutant strain exhibited significantly increased sensitivity to serum killing compared to the wild-type SC096 strain (P < 0.01). The complemented strain restored the serum resistance phenotype. Error bars represent the standard deviations from three independent experiments.

Additional file 9. Proposed model of G1P formation and contributing to G. parasuis polysaccharide biosynthesis. Glucose 1-phosphate can be generated from glucose, galactose and endogenous (glycogen) or exogenous (dextrin) glycogen, followed by conversion to UDP-glucose and UDP-galactose via GalU and GalE respectively. Both UDP-sugars then may participate in LOS or CPS formation.

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Authors’ contributions
SF: performed research, analyzed data, wrote paper and funded research. AC: performed research. XW, ZP, HY: helped with experiment. SX: analyzed data. SF: performed research, analyzed data, wrote paper and funded research. All authors read and approved the final manuscript.

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