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Characterization and Proteome Analysis of Inosine 5-Monophosphate Dehydrogenase in Epidemic *Streptococcus suis* Serotype 2

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Abstract *Streptococcus suis* serotype 2 (SS2) is an important zoonotic pathogen that causes severe disease symptoms in pigs and humans. In the present study, we found one isogenic mutant lacking inosine 5-monophosphate dehydrogenase (IMPDH) ΔZY05719 was attenuated in pigs compared with the wild-type SS2 strain ZY05719. Comparative proteome analysis of the secreted proteins expression profiles between ZY05719 and ΔZY05719 allowed us to identify Triosephosphate isomerase (TPI) and glyceraldehyde phosphate dehydrogenase (GAPDH), which were down expressed in the absence of the IMPDH. Both of them are glycolytic enzymes participating in the glycolytic pathway. Compared with ZY05719, ΔZY05719 lost the ability of utilize mannose, which might relate to down expression of TPI and GAPDH. In addition, GAPDH is a well-known factor that involved in adhesion to host cells, and we demonstrated ability of adhesion to HEp-2 and PK15 by ΔZY05719 was significantly weakened, in contrast to ZY05719. The adhesion to host cells is the crucial step to cause infection for pathogen, and the reduction adhesion of ΔZY05719, to some extent illustrates the attenuated virulence of ΔZY05719.

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Introduction

Streptococcus suis serotype 2 (SS2) is an important zoonotic pathogen, which can cause meningitis and sepsis in humans, as well as various diseases including meningitis, arthritis, septicaemia, and sudden death in piglets [9, 16, 21]. In 1998 and 2005, it caused two large outbreaks of severe human infections in China [27], and it was identified as the most frequent cause of bacterial meningitis in adults in southern Vietnam [17]. In view of its hazardous to public health, the scientific community accord thorough concern with this organism, a great quantity of structure on putative virulence factors have appeared in the literature [3, 8, 20]. These involve the polysaccharidic capsule (CPS), muramidase-released protein (MRP), extracellular factor (EF), suilysin (SLY), etc. CPS is so far the only known essential virulence factor protecting the pathogen against phagocytosis [7, 22]. However, most encapsulated SS2 are non-virulent strains, and loss of capsular production might be beneficial to *S. suis* in the course of infective endocarditis [14]. Isogenic mutants lacking MRP, EF, and SLY are still virulent to young piglets, and many virulent isolates of SS2 lacking these factors have also been isolated from clinical cases [1, 4, 23]. Limited knowledge of structures associated with virulence of SS2, which may be hamper to find prove and commercially available subunit vaccine. Therefore, a more thorough understanding of other virulent factor(s) in SS2 is very important.

Inosine 5-monophosphate dehydrogenase (IMPDH) is an important virulent factor in *Streptococcus pyogenes*, *Escherichia coli*, *Bacillus cereus*, *Borrelia burgdorgeri*, and *Agrobacterium* [2, 12, 28, 31]. On the other hand, IMPDH enzyme has been characterized and validated as a molecular drug target in many apicomplexans [6]. We first found a protein encoded by 975 bp gene have IMPDH activity in SS2-H, and named the gene *impdh* (GenBank Accession No. DQ097893.1). Furthermore, in contrast to the parent SS2-H,

isogenic mutant lacking *impdh* SS2-ΔH was found to be attenuated in various animal models, including BALB/c mice, rabbit, and pig [30]. However, the mechanisms by which IMPDH affect SS2 virulence remain obscure. In the present study, we constructed another *impdh* mutant strain SS2-ΔZY05719, and evaluated the effects of the *impdh* deletion on the characteristics of ZY05719. In addition, proteome analysis was performed to obtain a more detailed picture of the global regulation of *impdh* in ZY05719.

Materials and Methods

Strains, Plasmids, Growth Conditions, and Primers

The *S. suis* strain ZY05719 was a highly virulent strain isolated from diseased pigs in Sichuan Province, China, in 2005 [29]. ZY05719 and its derivatives were grown at 37 °C in Todd-Hewitt (Difco Laboratories) broth with 2 % yeast extract (THY) or plated on THY agar. *E. coli* DH5α was used for cloning purposes and was grown in Luria–Bertani (LB) or plated on LB agar at 37 °C. When necessary, plate or broth was supplemented with antibiotic(s) at the following concentrations: chloramphenicol (*cat*), 4 μg mL^{-1} for *S. suis*, 8 μg mL^{-1} for *E. coli*; and spectinomycin, 100 μg mL^{-1} for *S. suis*, 50 μg mL^{-1} for *E. coli*.

pICI plasmid was stored in our laboratory [30], which was constructed by pSET4s, *cat*, upstream flanking region of *impdh* gene, and downstream flanking region of *impdh* gene (Supplementary Fig. 1). A specific primer set (Table 1) was used to analyze *impdh* and *cat*.

Construction of the *impdh* Knockout Mutant of ZY05719

To construct the *impdh* knockout mutant strain, the *impdh* gene was insertionally inactivated in *S. suis* serotype 2 strain ZY05719 with a *cat* resistance cassette by pICI plasmid, as previously described [30].

Growth Characteristics of *impdh* Knockout Mutant Strains

ZY05719 and *impdh* deletion mutant ΔZY05719 were separately inoculated into 100 mL THY broth, and incubated at 37 °C. Samples of culture were monitored at 1 h intervals with a spectrophotometer (Eppendorf, German). Absorbance was measured at 600 nm in Disposable cuvettes (Eppendorf, German). Un-inoculated THY broth served as blank control. The experiments were repeated three times.

Biochemical Characterization of ΔZY05719 and ZY05719

The comparison of biochemical characterizations was performed as previously described [30] with a few modifications. Briefly, we analyzed the utilization of eighteen carbohydrates by bacterial micro-biochemical reaction tubes (HangZhou Microbial Reagent Co. Ltd), which containing Lactose, Salicin, Xylitol, Dextrin, Cellobiose, Sorbitol, Galactose, Maltose, Saccharobiose, Mannose, Serum inulin, Glucose, Mannitol, Raffinos, Arabinose, Glycerol, Melibiose, and Ribose, following manufacturer's directions.

Determination of ΔZY05719 Virulence in Pigs

Randomized groups of 3 conventional pigs (weight about 20 kg) were intravenous injected with 1 mL ZY05719 or ΔZY05719 at the following concentrations: 2×10^7 CFU mL^{-1} and 2×10^6 CFU mL^{-1}. All pigs were monitored daily over 10 days period for rectal temperature, mortality, and clinical signs. The animal experiments were conducted in accordance with the requirements of Jiangsu

Table 1 Oligonucleotide primers used in this study

Primers	Primers sequence (5′–3′)	Functions
impdh-F	AGTCTACGGTGGAGGTTTGTCA	For PCR assay
impdh-R	ACCTCAGCCGAATCAATAGCA	For PCR assay
cat-F	CAGTCGGCATTATCTCAT	For PCR assay
cat-R	GACAAGTAAGCCTCCTAA	For PCR assay
GAPDH-F	CTTGGTAATCCCAGAATTGAACGG	For real-time PCR
GAPDH-R	TCATAGCAGCGTTTACTTCTTCAGC	For real-time PCR
TPI-F	ATCTGGGCAATCGGTACTGG	For real-time PCR
TPI-R	TGAACGCGAACTTTGTCTGC	For real-time PCR
EF-Tu-F	TATGCTCACATCGACGCTCC	For real-time PCR
EF-Tu-R	CGAGTTTGTGGCATTGGACC	For real-time PCR
16S rRNA-F	GTTGCGAACGGGTGAGTAA	For real-time PCR
16S rRNA-R	TCTCAGGTCGGCTATGTATCG	For real-time PCR

Province on the review of laboratory animal administration.

Precipitation of Extracellular Proteins

The extracellular proteins in supernatants were precipitated by trichloroacetic acid by the method described previously [29]. The protein content was determined using the PlusOne 2-D Quant Kit (GE Healthcare) following manufacturer's directions.

Two-Dimensional Gel Electrophoresis and Image Generation

Dissolved 500 μg protein in 250 μL rehydration/sample buffer (8 mol L^{-1} urea, 2 % CHAPS, 0.2 % Bio-Lyte 4/7 ampholyte, 50 mmol L^{-1} DTT, and 0.01 % bromophenol blue; BioRad). Proteins were adsorbed onto an 11 cm Immobiline DryStrip (IPG, pH range 4–7; BioRad), and isoelectric focusing (IEF) was performed in the BioRad PROTEAN IEF cell. 12 h of passive rehydration preceded zone electrophoresis at 17 °C under the following conditions: S1 250 V 30 min; S2 500 V 30 min; S3 1,000 V 1 h; S4 4,000 V 1 h; S5 8,000 V 2.5 h; and S6 8,000 V 30,000 V h. Before the second dimension, strips were equilibrated for 2 × 15 min in equilibration buffer (6 mol L^{-1} urea, 2 % SDS, 30 % glycerol, and 375 mmol L^{-1} Tris–HCl pH 8.8), containing 2 % DTT and 2.5 % iodoacetamide, respectively. The proteins were separated in 12.5 % polyacrylamide gel without a spacer gel. Electrophoresis was performed at 5 mA per gel for 30 min and then at 10 mA per gel until the tracking dye reached the bottom of the gels. All gels were stained with colloidal Coomassie Blue G-250 (CBB). Three replicates were run for all samples of each strain and then analyzed by the software Image Master Platinum 7.0 (GE Healthcare). The normalized protein amount for each protein spot was calculated as the ratio of that spot volume to the total spot's volume on the gel. Both Student *t* test (*P* value <0.05) and a threshold of 1.5-fold expression changed spot could attract our interest.

Mass Spectrometry Analysis of Protein and Database Searches

Coomassie Blue-stained protein spots of interest were cut out from the gels and sent to the GuangZhou FitGene Biological Technology Co. Ltd for trypsin in-gel digestion and MALDI-TOF-MS analysis. Protein spots with a low Mascot score were analyzed by MALDI-TOF/TOF-MS. Data from MALDI-TOF-MS and MALDI-TOF/TOF-MS analysis were used against the NCBInr protein database using MASCOT (http://www.matrixscience.com) with the parameter settings of trypsin digestion, one max missed cleavages, variable modification of oxidation (M), and peptide mass tolerance for monoisotopic data of 50 ppm. Peptide mass fingerprinting (PMF) data were searched in MASCOT for sequence matches. The probability score for the match, molecular weight (MW), isoelectric point (pI), number of peptide matches, and percentage of the total translated ORF sequence covered by the peptides were analyzed for confident spot identification.

RNA Manipulation and Quantitative Real-Time PCR

Total RNA was extracted from logarithmic phase bacterial with an E. Z. N. A.™ bacterial RNA kit (Omega, USA) following manufacturer's protocol. cDNAs were reverse transcribed with a PrimeScript RT-PCR kit (Takara, Dalian, China). Two-step relative quantitative and real-time PCR (qRT-PCR) were performed to analyze mRNA levels. Relative expression ratios of selected genes were normalized to the expression of a single housekeeping gene (16S rRNA), and fold changes were calculated as described by Livak and Schmittgen [15]. The specific primers used for the qRT-PCR assays are listed in Table 1. We utilized the SYBR Premix Ex Taq™ kit (Takara, Dalian, China) following manufacturer's instructions to analyze cDNA content. Assays were carried out in triplicate and data were analyzed by a two-tailed, paired *t* test. The ABI 7500 RT-PCR system was used for relative qRT-PCR.

Cell Adhesion Assays

The adhesion assay to quantify total cell-associated bacteria was performed as previously described [25], with some modifications. Briefly, bacteria were collected by centrifugation, washed twice with PBS, and resuspended in fresh cell culture medium without antibiotics. Confluent PK15 or HEp-2 cell monolayers were inoculated with bacteria suspension with a multiplicity of infection (MOI) of 100, and incubated for 3 h at 37 °C with 5 % CO_2. The monolayers were then vigorously washed 5 times with PBS to eliminate nonspecific bacterial attachment. The plates were then incubated for 10 min at 37 °C in the presence of 200 μL of 0.025 % trypsin-0.03 % EDTA. Next, 800 μL of ice-cold cell culture medium was added, and cells were disrupted by repeated pipetting to liberate cell-associated bacteria. Serial dilutions of this cell lysate were plated onto THY agar and incubated overnight at 37 °C, after which the bacteria were counted. All assays were repeated at least three times, and data were expressed as mean (and standard deviation). Data were analyzed by a two-tailed, unpaired *t* test to compare the adhesion capacities of several *S. suis* strains.

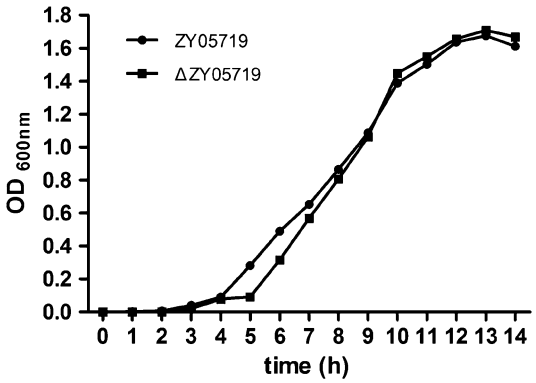

Fig. 1 Comparison of the growth curves of ΔZY05719 and ZY05719. Bacteria were cultured in THY at 37 °C. Absorbance at 600 nm was measured at intervals. Results shown are representative of three independent experiments

Results

Growth Rates of ZY05719 and ΔZY05719

An isogenic *impdh* knockout mutant ΔZY05719 was confirmed by PCR (Supplementary Fig. 2). The OD_{600nm} values of bacterial cultures showed that the growth rates of ΔZY05719 was almost identical to ZY05719 (Fig. 1).

Biochemical Characterization of ZY05719 and ΔZY05719

Results revealed that ZY05719 could not utilize Xylitol, Sorbitol, Serum inulin, Mannitol, Arabinose, Glycerol, and Ribose. In addition to above-mentioned carbohydrates, ΔZY05719 did not utilize mannose (Supplementary Table 1).

Virulence of ZY05719 and ΔZY05719 in Pigs

When three pigs were infected with 2×10^7 CFU ΔZY05719, two pigs showed clinical signs of *S. suis* two infection, including hyperthermy (above 41.1 °C), depression, and arthritis. The two pigs returned to normal rectal temperature 4 days post-infection (PI), one of the diseased died 5 days PI, and the another gradually recovered. In contrast, all three pigs infected with 2×10^7 CFU ZY05719 showed hyperthermy (above 41.5 °C), two of them died 48 h PI. The reminder returned to normal rectal temperature 6 days PI, but persistently presented arthritis for 7 days. Two of three pigs infected with 2×10^6 CFU ΔZY05719 developed hyperthermy (41.0 °C) 72 h PI, and they all returned to normal rectal temperature 4 days PI. Meanwhile, two of the three pigs infected with 2×10^6 CFU ZY05719 showed hyperthermy (above 40.5 °C) 24 h PI, one of them died 48 h PI, and the sick another returned to normal rectal temperature 72 h PI. These results suggested that ΔZY05719 was less virulent to pig than the wild strain ZY05719.

Comparative Proteomics

The representative two-dimensional gel electrophoresis and images of secreted protein of ZY05719 and ΔZY05719 are provided in Fig. 2. The majority of proteins were distributed in the range of pI 4–7. A total of five secreted protein spots were significantly different between ZY05719 and ΔZY05719, and they were identified to three individual proteins by MALDI-TOF-MS or MALDI-TOF/TOF-MS analysis. The probability score for the match, MW, pI, number of peptide matches, and the percentage of the total translated ORF sequence covered by the peptides were

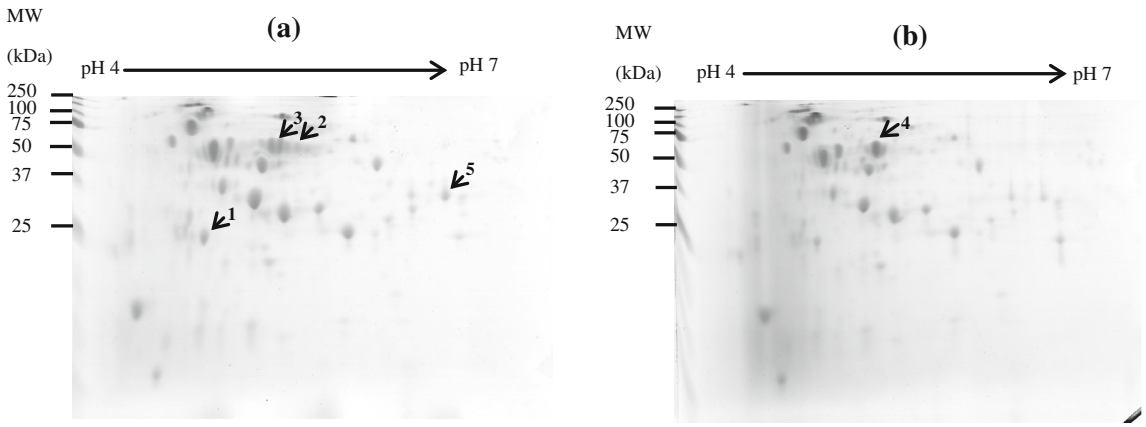

Fig. 2 2-DE proteome map (11 cm IPG strip, pH 4–7) of secreted protein from ZY05719 (**a**) and ΔZY05719 (**b**). The gel spots were encoded by the protein number, which was assigned based on their similar locations on different gels. *Arrow heads* represent protein spots with a significantly ($P < 0.05$) increased amount in each strain

Table 2 Protein spots identified by MALDI-TOF-MS or MALDI-TOF/TOF-MS

Spot number	Protein identified[a]	BLASTX similarity matched protein/species	Theoretical MW (Da)/pI	Experiment MW (Da)/pI[b]	Mascot Score[c]	No. of Petides matched[d]	Coverage (%)[e]	Fold change[f]	
								Mean	*P* value
1	gil146318185	TPI/*S. suis*	26,900/4.68	24,000/4.8	511	6	33	2.51	0.0004
2	gil395580687	Translation elongation factor Tu/*S. suis*	40,000/4.67	50,000/5.5	642	10	37	2.31	0.0132
3	gil253751367	Elongation factor Tu/*S. suis*	44,000/4.87	50,000/5.3	696	11	42	2.07	0.0143
4	gil253751367	Elongation factor Tu/*S. suis*	44,000/4.87	50,000/5.3	679	12	48	1.64	0.0221
5	gil223,933805	Glyceraldehyde-3-phosphate dehydrogenase, type I/*S. suis*	35,900/5.46	33,000/6.7	430	7	26	2.01	0.0028

[a] Gi number in NCBI

[b] Observed MW and pI of protein spot in the 2-D gel

[c] Macot score obtained for the PMF

[d] Number of peptides that match the predicted protein sequence

[e] Percentage of predicted protein sequence covered by matched peptides

[f] Threshold of 1.5-fold expression significantly changed spot

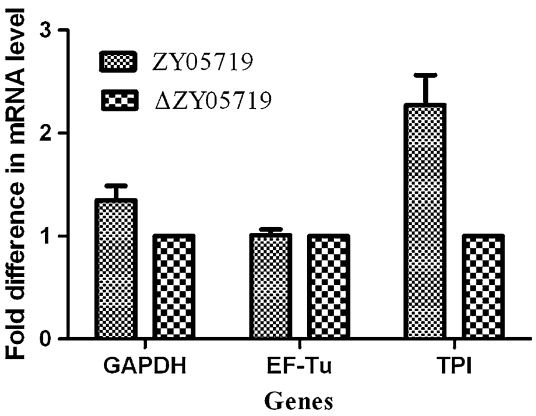

Fig. 3 The expression levels of GAPDH, EF-Tu, and TPI mRNA between ZY05719 and ΔZY05719. The value of the genes in ΔZY05719 as 1.0. The comparative cycle threshold method ($2^{-\Delta\Delta CT}$ method) was used to analyze the relative change in gene expression ratios of normalized genes by housekeeping gene (16S rRNA gene)

used as confidence factors in protein identification. The results are summarized in Table 2.

Confirmation of Comparative Proteomics Results by Transcriptional Analysis

The qRT-PCR results validated the results of comparative proteomic analysis. As shown in Fig. 3, ZY05719 had 1.34 times higher glyceraldehyde phosphate dehydrogenase (GAPDH) mRNA ($P < 0.05$) and 2.27 times higher TPI mRNA ($P < 0.05$) than ΔZY05719. Meanwhile, there was no significant difference in EF-Tu mRNA ($P = 0.74$) between ZY05719 and ΔZY05719.

Contribution of *impdh* to in Vitro Adhesion

To assess the contribution of *impdh* to in vitro adhesion, the capacity of adhesion to PK15 and HEp-2 cells was compared between ZY05719 and ΔZY05719. As shown in Fig. 4, there was significant reduction in the adherence of ΔZY05719 compared with the wild-type strain ZY05719.

Discussion

IMPDH catalyzes the pivotal step in guanine nucleotide biosynthesis, the conversion of IMP to XMP with the concomitant reduction of NAD^+ in a reaction that involves a covalent intermediate E-XMP^*. In our previous study [30], we found the deletion of *impdh* gene was crucial to the virulence of SS2-H, however, the reason for the phenomenon was not clear. To ascertain the influence on virulence of *impdh* as well as the perhaps modulate mechanism of *impdh* gene, we constructed another *impdh* deletion strain ΔZY05719. Our previous study revealed that SS2-ΔH was slower in growth than SS2-H, however, we demonstrated the growth rates of ΔZY05719 was almost identical to ZY05719 in the present study. Hence, the influence of the deletion of *impdh* to the growth of various SS2 strains might be different. On the other hand, the virulence of ΔZY05719 was attenuated in pigs, and the same result was found in SS2-ΔH.

Bacteria secrete proteins that fulfill a variety of functions to insure bacterial survival in their respective environments. These proteins could be associated with cell adhesion and invasion, as well as survival and proliferation in eukaryotic

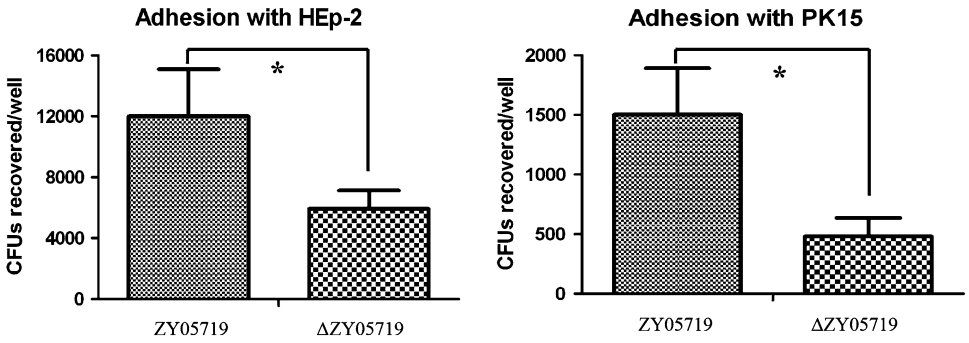

Fig. 4 Effect of *impdh* inactivation on the adherence to HEp-2 or PK15 cells. An *asterisk* indicates a significant difference ($P < 0.05$) between different strains

hosts [24]. Baums and Valentin-Weigand [3] published a complete and comprehensive review of *S. suis* secrete proteins. Three virulence associated proteins, MRP, EF [26], and SLY [10] could be extracted from the supernatant of SS2 cultures and evaluated for use as subunit vaccines against SS2. To elucidate the precise regulatory mechanism of IMPDH on virulence in *S. suis*, we compared the secret proteins expression profiles of ZY05719 and the mutant. This analysis revealed four up-regulated proteins and one down-regulated protein in ZY05719. Two out of four up-regulated and one down-regulated protein were all EF-Tu; furthermore, the difference of EF-Tu mRNA between ZY05719 and ΔZY05719 was not significant. So we presumed there was no difference in expression of EF-Tu between ZY05719 and ΔZY05719.

Spot 1 is TPI, which is a glycolytic enzyme that catalyzes the interconversion of dihydroxyacetone phosphate and D-glyceraldehyde-3-phosphate. The reaction is very efficient and requires neither cofactors nor metal ions. Spot 5 is GAPDH, which is an enzyme of the glycolytic pathway responsible for the phosphorylation of GAP to generate 1,3-bisphosphoglycerate, and is usually found in the bacterial cytoplasm. We found the existence of GAPDH in the supernatant of SS2 cultures, and many proteins could be excised from bacterial cytoplasm as well as supernatant of culture. TPI and GAPDH are glycolytic enzyme, and participating glycolytic pathway. Compared with the parent strain ZY05719, ΔZY05719 lost the ability of utilize mannose. So, we presumed the down expression of TPI and GAPDH in ΔZY05719 disturbed its utilization of mannose. And in our previous study, SS2-ΔH could not utilize maltose, lactose, raffinose, mannose, and glucose compared with SS2-H. GAPDH and IMPDH were identified as plasminogen receptors (PlgR) on the surface of *Staphylococcus aureus* (*S. aureus*) [18, 19]. *S. aureus*-bound plasminogen can be converted to plasmin by host plasminogen activator (PA) or staphylococcal PA. Plasmin generated on bacterial surface may enhance bacterial to penetrate tissues during infections. GAPDH and TPI were also identified as PlgRs in Streptococci [11, 13], but whether IMPDH was PlgR in SS2 had not been reported to our knowledge.

Transposon mutants deficient in the expression of GAPDH were attenuated in the adhesion to embryonic bovine tracheal cells and porcine tracheal rings, suggesting that GAPDH is involved in adhesion to host cells [5]. Finally, we compared the adhesion of ZY05719 and ΔZY05719 with HEp-2 and PK15 cell, respectively. We found significant decline of adhesion with HEp-2 and PK15 by ΔZY05719. Adherence of pathogenic bacteria to the mucosal surface is considered to be a criticality step in the infection process, so we presumed the attenuated virulence of ΔZY05719 was the result of its reduction of adhesion with HEp-2 and PK15.

In conclusion, the present study provided insight into the role of the IMPDH in the characterization and virulence of ZY05719. Our results demonstrated that the influence of IMPDH on the growth SS2-H and ZY05719 were not identical, but the influence of IMPDH on the virulence of SS2 was ascertained. In addition, the deletion of *impdh* gene resulted in down expression of TPI and GAPDH, which might be the reason for negative utilize of mannose by ΔZY05719. Furthermore, the down expression of GAPDH of ΔZY05719 to some extent caused the declined adhesion to HEp-2 and PK15 by ΔZY05719.

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