VIRULENCE
2020, VOL. 11, NO. 1, 825–838
https://doi.org/10.1080/21505594.2020.1782088

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Screening for phagocytosis resistance-related genes via a transposon mutant library of *Streptococcus suis* serotype 2

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ABSTRACT

Streptococcus suis: serotype 2 (SS2) is a serious zoonotic pathogen which causes symptoms of streptococcal toxic shock syndrome (STSS) and septicemia; these symptoms suggest that SS2 may have evade innate immunity. Phagocytosis is an important innate immunity process where phagocytosed pathogens are killed by lysosome enzymes, reactive oxygen, and nitrogen species, and acidic environments in macrophages following engulfment. A previously constructed mutant SS2 library was screened, revealing 13 mutant strains with decreased phagocytic resistance. Through inverse PCR, the transposon insertion sites were determined. Through bioinformatic analysis, the 13 disrupted genes were identified as *Cps2F*, 3 genes belonging to ABC transporters, *WalR, TehB, rpiA*, S-transferase encoding gene, *prs, HsdM*, GNAT family N-acetyltransferase encoding gene, *proB*, and upstream region of *DnaK*. Except for the capsular polysaccharide biosynthesis associated *Cps2F*, the other genes had not been linked to a role in anti-phagocytosis. The survival ability in macrophages and whole blood of randomly picked mutant strains were significantly impaired compared with wild-type ZY05719. The virulence of the mutant strains was also attenuated in a mouse infection model. In the *WalR* mutant, the transcription of *HP1065* decreased significantly compared with wild-type strain, indicating *WalR* might regulated *HP1065* expression and contribute to the anti-phagocytosis of SS2. In conclusion, we identified 13 genes that influenced the phagocytosis resistant ability of SS2, and many of these genes have not been reported to be associated with resistance to phagocytosis. Our work provides novel insight into resistance to phagocytosis, and furthers our understanding of the pathogenesis mechanism of SS2.

ARTICLE HISTORY
Received 25 December 2019
Revised 17 April 2020
Accepted 7 June 2020

KEYWORDS
Streptococcus suis serotype 2; transposon mutant library; phagocytosis resistance; *WalR*; *HP1065*

Introduction

Streptococcus suis serotype 2 (SS2) is a zoonotic pathogen that has caused severe diseases in humans and pigs, including streptococcal toxic shock syndrome (STSS), sudden death, septicemia, and meningitis; SS2 has resulted in great economic losses in the porcine industry [1,2]. Since SS2 infection can cause STSS, it is plausible that SS2 could successfully evade innate immunity. Phagocytosis is a crucial step of host immunity, and hence, the phagocytosis resistance ability of SS2 has attracted our attention. A transposon mutant library of ZY05719 was previously constructed in our laboratory [3]. This mutant library contained 2400 strains of SS2 mutants in total. The *mariner Himar1* transposon was randomly inserted into the genome, and the transposon distribution within the SS2 genome was shown to be random without any hot spots [3]. Transposon mutants have been used to screen for virulence-associated genes by generating altered phenotypes compared to wild-type strains [4–7].

Macrophages have evolved to defend against pathogens by phagocytosis and eliminate intracellular pathogens by lysosomal proteases, phagosome acidification, reactive oxygen and nitrogen species (ROS and RNS), and antimicrobial peptides. However, pathogens have also developed ways to counteract elimination by host cells. For example, pathogens can defend against opsonophagocytosis and other forms of phagocytosis of macrophages. Additionally, they are able to resist lysosome enzymes, reactive oxygen, and nitrogen species (ROS and RNS), and overcome host nutritional limitations by the host [8]. In SS2, capsular polysaccharide plays an important role in phagocytosis resistance [9]. Suilysin is another well-known phagocytic resistance factor that contributes to opsonophagocytosis resistance, phagocytosis resistance, and intracellular survival within phagocytes of SS2 [10]. Sialic acid is a component of the SS2 capsule. Biosynthesis of sialic acid is encoded by *neuC* gene. Deletion of *neuC* gene results in decreased phagocytosis resistance of SS2 [11].

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A novel transglutaminase of SS2 was shown to be associated with phagocytic resistance [12]. The factor H binding protein (Fhb) of SS2 could bind to human factor H, thus blocking the deposition of complement on the bacteria, which further contributes to phagocytosis resistance ability of SS2 [13].

In this study, we screened 1100 mutant strains from a previously generated mutant library for mutants that led to an increased rate of phagocytosis compared to the wild-type strain ZY05719. We identified 13 phagocytosis susceptibility mutants which could possibly contribute to attenuated virulence. Only one of the 13 genes (capsule biosynthesis gene) has previously been reported for involvement in phagocytosis resistance [14], and the rest remains to be elucidated through further study. *WalR* was identified in our screen and was reported to be involved in cell wall biosynthesis [15,16]. Cell wall synthesis-related genes were examined, and *LysM* transcription was shown to increase, along with a corresponding decrease in *HP1065* transcription relative to wild-type ZY05719; this might be implicated in the impaired phagocytosis resistance ability of the *WalR* mutant strain.

Materials and methods

Bacterial strains, cell lines and culture conditions

All the strains and plasmids used in this study are listed in Table 1. The wild-type SS2 strain ZY05719 was isolated from a diseased pig from an SS2 outbreak in Ziyang, China. A mutant strain library based on strain ZY05719 was previously constructed in this lab. Briefly, the pMar4 s plasmid containing the TnYLB-1 transposon was transformed into ZY05719 by electroporation. The mutant pool was stored in 50% glycerol at −80°C. Both wild-type SS2 strains and mutant strains were cultured in Todd-Hewitt Broth (THB; Becton Dicksinson) liquid or agar media at 37°C. For growth assay, THB liquid media supplemented with 2% Yeast Extract (THY) was used. *Escherichia coli* DH5α was maintained in Luria-Bertani (LB) medium. When the pSET2 or pSET4s plasmids were transformed into DH5α and the SS2 mutant strain, the THB medium was supplemented with 50 μg/mL spectinomycin (Spc) or 100 μg/mL Spc, respectively.

Table 1. Bacterial strains and plasmids used in this study.

Names	charateristics[a]	source
strains		
ZY05719	Isolated from a diseased pig in Sichuan Province in China, 2005	conserved in this lab
DH5α	Host for recombinant plasmids cloning	purchased from Tiangen
Δ*MetQ*	Isogenic *MetQ* mutant strain of ZY05719	This study
CΔ*MetQ*	Complemented strain of Δ*MetQ*, Spc^r	This study
Δ*WalR*	Isogenic *WalR* mutant strain of ZY05719	This study
CΔ*WalR*	Complemented strain of Δ*WalR*, Spc^r	This study
Δ*HP1065*	Isogenic *HP1065* mutant strain of ZY05719	This study
C*LysM*	Overexpression strain of *LysM*, Spc^r	This study
plasmids		
pSET2	*E.coli-S.suis* shuttle vector, Spc^r	23
pSET4s	*E.coli-Streptococcus* shuttle cloning vector, Spc^r	22

[a]Spc^r, spectinomycin resistance

Raw264.7 macrophage cells derived from mouse Abelson murine leukemia virus-induced tumors were purchased from the American Type Culture Collection (ATCC). Cells were cultured in high-glucose Dulbecco's modified Eagle medium (DMEM) (Gibco, Invitrogen) supplemented with 10% fetal bovine serum (Gibco, Invitrogen) at 37°C under a 5% CO_2 humidified atmosphere.

Phagocytosis assay, intracellular survival assay and adhesion assay in macrophages

A phagocytosis assay was performed according to previous research with a few modifications [17]. In brief, wild-type and each mutant strain were cultured separately to an OD_{600} of 0.6 ~ 0.8, washed with phosphate-buffered saline (PBS) 3 times and resuspended in DMEM. Raw264.7 cells were cultured to approximately 3.0×10^5 cells/well in a 24-well plate for the phagocytosis assay. Each well of Raw264.7 cells was infected with different single mutant or wild-type strain at a multiplicity of infection (MOI) of 10:1. After incubation for 1 h at 37°C under 5% CO_2, the cells were washed with PBS 3 times and incubated with DMEM supplemented with 10 μg/mL penicillin G and 100 μg/mL gentamicin for 1 h to kill extracellular bacteria. The cells were washed with PBS three times, and 1 mL of ddH_2O was added to each well to lyse the cells. For the initial semiquantitative screen, 900 μL of ddH_2O was removed from the wells and 900 μL of Semisolid Todd-Hewitt agar (THA) were added. The phagocytosed mutant bacteria were compared with the phagocytosed wild-type strain in the 24-well plates. The mutant strains with obviously increased CFUs in the assay were verified with a quantitative phagocytosis assay, which was similar to the initially performed phagocytosis assay except that 1 mL of ddH_2O containing the intracellular bacteria, was serially diluted and plated on THA. The phagocytosed bacteria were counted, and significant increases in CFU ($P < 0.05$) of mutant strains relative to the wild-type control strain indicated attenuated phagocytosis resistance. Assays were performed independently in triplicate.

An intracellular survival assay was conducted as previously described [18]. In brief, the initial steps were the same as the phagocytosis assay. After

incubation with antibiotics, cells were lysed at different timepoints of cells (1 h and 3 h), as described in the phagocytosis assay and counted by serial dilution. The intracellular survival rate was calculated as $CFU_{3\ h}/CFU_{1\ h} \times 100\%$. The assay was performed independently three times.

The macrophage adhesion assay was conducted as follows. Raw264.7 cells were pretreated with 1 μg/mL cytochalasin D (Gene Operation) for 30 min at 37°C under 5% CO_2 to inhibit actin polymerization. Cells were infected with bacteria at an MOI of 10:1 for 2 h. The wells were then washed with PBS for 5 times. The cells were lysed with 1 mL ddH_2O, and the adherent bacteria were plated on THA at a suitable dilution. The colonies derived from bacteria attached to Raw264.7 cells were counted. The assay was performed three independent times.

Defining the transposon insertion sites

Chromosomal DNA was extracted from mutant bacteria with a TIANamp Bacteria DNA Kit (TIANGEN) following the manufacturer's instructions. The genomes of mutant strains were digested with *TaqI* (Takara) at 65°C for 5 h as per the manufacturer's protocols. The fragmented genome was then extracted with a MiniBEST DNA Fragment Purification Kit (Takara). Fragments were circularized with T4 DNA Ligase (Takara) at 16°C overnight. The ligation products were purified and acted as the inverse PCR templates. Inverse PCR was conducted to detect the flanking region of the transposon insertional site. Primers oIPCR1 and oIPCR2 were used in this assay and were listed in Table 2. The PCR products were purified and sequenced. BLAST was used to identify the insertional genes from the sequence data. Some BLAST results showed continuous genes that required further confirmation by PCR. The primers for each gene are listed in Table 2.

Bactericidal assay

The assay was modified based on a previous report [19]. ZY05719 and the mutants were grown to mid-exponential phase and washed 3 times with PBS. The bacteria were then resuspended in PBS to an OD_{600} of 0.1. A total of 100 μL of bacteria were added to 900 μL of whole pig blood, and incubated together at 37°C for 3 h. The survival rate was calculated by $CFU_{3\ h}/CFU_{0\ h} \times 100\%$. Each assay was performed independently in triplicate.

Survival curve of mice infected with transposon mutant strains

To determine the virulence of the transposon mutants that showed decreased phagocytosis resistance ability, the virulence of M319 (*MetQ*), M431 (*WalR*), and M137 (ABC transporter) was determined in mice. Each group contained eight specific pathogen-free (SPF) Balb/c mice (female, 4 weeks old). The aforementioned bacteria were cultured along with wild-type ZY05719 to log phase; the mice were challenged via intraperitoneal injection at a dose of 3×10^8 CFU/mouse. The survival rate was monitored every day for 7 d.

To validate the virulence of the mutant strains, the mice were challenged with two knockout strains Δ*MetQ* and Δ*WalR*, as well as their complemented strains CΔ*MetQ* and CΔ*WalR*. The method was the same as previously described.

Growth assay

Mutants with decreased phagocytic resistance and ZY05719 were cultured to an OD_{600} of 0.6 in THY and inoculated into fresh THY (1:100 dilution). Bacteria were cultured at 37°C with vigorous shaking at 180 rpm. The OD_{600} was measured hourly for 12 h, and then the 24-h OD_{600} was measured. All the growth kinetics were measured with three biological replicates. Data were shown as the means with standard deviations.

Transmission electron microscopy

Capsules of ZY05719, M431, and M604 were observed with transmission electron microscopy (TEM) as described previously [20]. The strains were cultured to an OD_{600} of 0.6–0.8 and centrifuged at 5000 rpm for 5 min, washed three times with PBS and fixed in 2.5% glutaraldehyde overnight. The samples were then dehydrated with propylene oxide for 10 min and embedded in epoxy resin. After fixation, the sections were observed with the Hitachi H-7650 system. The CPS thickness of each strain was measured in 10 different bacteria by Image J software according to the graphical scale. The capsule thicknesses were shown in the histogram.

RNA manipulation and real-time quantitative PCR (qPCR)

Wild-type ZY05719 and M431were grown to exponential phase at an OD_{600} of 0.6–0.8, and the total RNA was extracted. The RNA extraction was conducted using an E.Z.N.A. Bacterial RNA Kit (Omega). cDNA was synthesized using HiScript II Q RT SuperMix for

Table 2. Primers used in this study.

Name	Oligonucleotide sequence (5′–3′)[a]	Product
olPCR1	gcttgtaaattctatcataattg	flanking region of transposon
olRCR2	agggaatcatttgaaggttgg	flanking region of transposon
detection of inserted gene		
111 f	ttacacttctgcgataatgggaata	ribose 5-phosphate isomerase
111 r	atgaaaatcattaacacgacaaata	ribose 5-phosphate isomerase
137 f	gtgcctgcggatgttttggcttccg	BCAA transporter
137 r	ttacatttcaaaaacttcgctctcc	BCAA transporter
71 f	ttaattatcaattgaatgatatttc	S-transferase
71 r	tgacagaatatacaaaaccactcgt	S-transferase
248 f	atgattgaaattcactatctt	prs
248 r	ctaatctagtgtttgtaaata	prs
678 f	atgaaggagaatattaaacaaatgg	HsdM
678 r	tcagaaaaatctaatttgaaaggag	HsdM
431 f	ttaagtaccctctatttgataacca	WalR
431 r	atgtcaaggattttggttgtaga	WalR
535 f	ctattttagtttttgccaataagct	hypothetical protein
535 r	atgagtaaatccatcatttgggaac	hypothetical protein
319 f	acaactgttcgagtaggtgtgat	MetQ
319 r	ttaccaaactggctcatccataccg	MetQ
604 f	atgagaacagtttatattattggtt	Cps2F
604 r	ttatcctttaaacaacttctcatac	Cps2F
743 f	ttatcctttctggtgcaaag	GNAT family N-acetyltransferase
743 r	atgcagattgatcctatacgttt	GNAT family N-acetyltransferase
686 f	ttatccttttttcctagccag	TehB
686 r	atgactgttttagttccttacaaac	TehB
1020 f	atgacagaaaaaacaattgtattta	proB
1020 r	ctataagctcacccaatcgtt	proB
1051 f	tagtcctcagttattggcaccagag	upstream of DnaK
1051 r	agcctagtttaatgacatttcggtc	upstream of DnaK
qPCR		
parC-F	tggagatgcacggaaacaa	
parC-R	ctcgatgtcagccaagagatag	
PcsBF	gcacaacaagcagaagctca	
PcsBR	aatatctgccgccaatgtct	
LysM2173 F	cgaattgggtgatgtgacag	
LysM2173 R	ctgctgcatcttcagcactc	
LysM0229 F	catatgcgtcgcaagagaaa	
LysM0229 R	acagtacgtggagcccattc	
LytBF	gcatggggaacgtcatactt	
LytBR	tgcttcttctcaccatcacg	
1065 f	agaaggcaccagagacgaaa	
1065 r	agttggggctggaacttctt	
murGF	acgtgacgctcaatctcctt	
murGR	aataccgttcccatcaccaa	
Pbp1aF	atcagggaacctgcaatacg	
Pbp1aR	ggagcagaaaaacgttcgag	
Pbp1bF	attcggatggtagcttggtg	
Pbp1bR	cttaggcacaaccccattgt	
PgdAF	tgcccttctgctaccattct	
PgdAR	cctttgatgatggacccaat	
MurAF	agcttcttccgtcacctcaa	
MurAR	tggtggagatgtcttggtca	
q431 f	acaattttcccacgtcgttt	
q431 r	tagcggggcagtttatatgg	
construction of recombinant plasmid		
CM431 F	aaacgacggccagtgaattcttcatcattggggtctggat	WalR and its promoter
CM431 R	cgggtaccgagctcgaattcatggatggagtgttggaagc	WalR and its promoter
CM319 F	ccgggtaccgagctcgaattcgacaggtttggatcctagagatggt	MetQ and its promoter
CM319 R	aacgacggccagtgaattccgccacatccagcaaacc	MetQ and its promoter
CMLysMF	tgcatgcctgcaggtcgacgcttggctgatggtattg	LysM2173 and its promoter
CMLysMR	gggatcctctagagtcgactattatggcttcgggcta	LysM2173 and its promoter
WALR1	gggatcctctagagtcgactccgttcttaattcatcg	upstream of WalR
WALR2	atagaactttacgaggaggtatggggattgccagaaaa	upstream of WalR
WALR3	ttttctggcaatccccatacctcctcgtaaagttctat	downstream of WalR
WALR4	tgcatgcctgcaggtcgacataacatcagccacagca	downstream of WalR
MetQ1	aaaacgacggccagtgaattcgcttgccgaatcaatgacacca	upstream of MetQ
MetQ2	acagttaatttaaggagatcattttaatgtaaataggttgaggactgggc	upstream of MetQ
MetQ3	gcccagtcctcaacctatttacattaaaatgatctccttaaattaactgt	downstream of MetQ
MetQ4	ccgggtaccgagctcgaattcgaacgggatgaatttgaactgg	downstream of MetQ
HP1065-F1	gggatcctctagagtcgaccctcctcgtcttctcgtc	upstream of HP1065
HP1065-F2	tgcctctttattatttcataatggtttctataagtcctaatcatatttta	upstream of HP1065
HP1065-F3	taaaatatgattaggacttatagaaaccattatgaaataataaagaggca	downstream of HP1065
HP1065-F4	tgcatgcctgcaggtcgacaactccgtgtcgcttcta	downstream of HP1065

[a]Underlined portions indicate restriction enzyme recognition sites of primers.

qPCR (+gDNA wiper) (Vazyme) following the manufacturer's instructions. mRNA levels were determined with a SYBR Premix Ex TaqTM kit (TaKaRa) in the ABI Step One Plus Real-Time PCR System. Primers are listed in Table 2. The *parC* gene was used as a reference [21]. Relative expression of the target gene was calculated using the $2^{-\Delta\Delta Ct}$ method. The experiment was repeated three times independently.

Construction of knockout strains, complemented strains and overexpression strain

To obtain a knockout strain of *MetQ, WalR,* and *HP1065* (Δ*MetQ*, Δ*WalR*, Δ*HP1065*), homologous recombination method was conducted as previously reported [22]. Briefly, the flanking regions of the corresponding genes were amplified (PrimeSTAR HS, Takara) and fused by PCR with the primers listed in Table 2. The fusion fragments were ligated to the pSET4s [22] shuttle vector (ClonExpress® MultiS One Step Cloning Kit, Vazyme). Recombinant plasmids were transformed into ZY05719 and the bacteria were cultured on TH agar supplemented with 100 μg/mL spectinomycin at 37°C. The colonies grown on the plate were inoculated and cultured in THB at 28°C for 10 generations. The knockout strains were spectinomycin susceptible and confirmed by PCR.

Complementation strains of Δ*MetQ* and Δ*WalR* were constructed by using the shuttle vector pSET2 [23]. Regions containing whole open reading frames (ORFs) as well as putative promoters and terminators of the corresponding genes were amplified using primers CM319 F/R and CM431 F/R. The products were then ligated to the pSET2 plasmid. The recombinant vectors pSET2-319 and pSET2-431 were, respectively, electroporated into Δ*MetQ* and Δ*WalR* competent cells to generate the complementation strains CΔ*MetQ* and CΔ*WalR*.

To obtain the *LysM2173* overexpression strain (C*LysM*), pSET2-*LysM2173* was first constructed by amplifying *LysM2173* as along with the putative promoter and terminator with the primers CM*LysM*F/R, and then ligating the PCR product to the pSET2 vector. The recombinant plasmid pSET2-*LysM2173* was transformed into the wild-type SS2 ZY05719.

Ethics statement

All animal experiments were conducted according to the guidelines of the Institutional Animal Care and Use Committee of Nanjing Agricultural University (Nanjing, China) and approved by the Committee of the Faculty of Veterinary Science of Nanjing Agricultural University (Permit Number: PZ2019132). SPF Balb/c mice were purchased from the Comparative Medicine Center of Yangzhou University and kept in ventilated cages at room temperature of 25°C. After all experiments, surviving mice were anesthetized with isoflurane and euthanized by CO_2.

Statistical analysis

Significant differences were determined using unpaired two-tailed Student's *t*-test with GraphPad Prism 5 software. Each experiment was performed as three independent repeats. For all tests, a P value < 0.05 was considered statistically significant. Data were expressed as the means with a standard error of the mean (SEM) or the means with standard deviations (SD).

Results

Identification of mutant strains that have decreased phagocytosis resistance capability from mutant library

The initial screening was semiquantitative, and the phagocytosis assay was conducted as such to increase throughput. In the last step, 900 μL of the ddH_2O which lysed the cells were discarded and 900 μL semi-solid THB were added to the wells directly and plates were cultured at 37°C overnight. Colonies were visualized in the 24-well plates to compare with wild-type ZY05719. The M229 mutant strain had no obvious difference in colony numbers compared with ZY05719, whereas the M248 showed obviously increased CFU relative to ZY05719 in the semi-solid THB (Figure 1(a)). After screening approximately 1100 mutant strains from the mutant pool, 13 mutant strains were identified that had a decreased phagocytosis resistance phenotype. These 13 mutants were verified by a quantitative phagocytosis assay to determine the exact phagocytic rate compared with the wild-type strain (Figure 1(b)). As shown in Figure 1(b), the mutants of interest exhibited increased phagocytosis rates relative to ZY05719 to different degrees from twofold to 29-fold ($P < 0.01$). *Cps2F* mutant (M604) showed the most obvious change in phagocytosed bacteria compared to the wild-type strain. Since capsular polysaccharide (CPS) is an important anti-phagocytic factor, this result was in accordance with the previous report.

The transposon insertion site in the SS2 genome was examined by amplifying the flanking region of the transposon with primers oIPCR1/2. The PCR products were sequenced, and the sequences were aligned by BLAST. The disrupted genes are listed in Table 3. Some sequencing results showed two or more adjacent genes because of the random digest by *TaqI* and the following circularization,

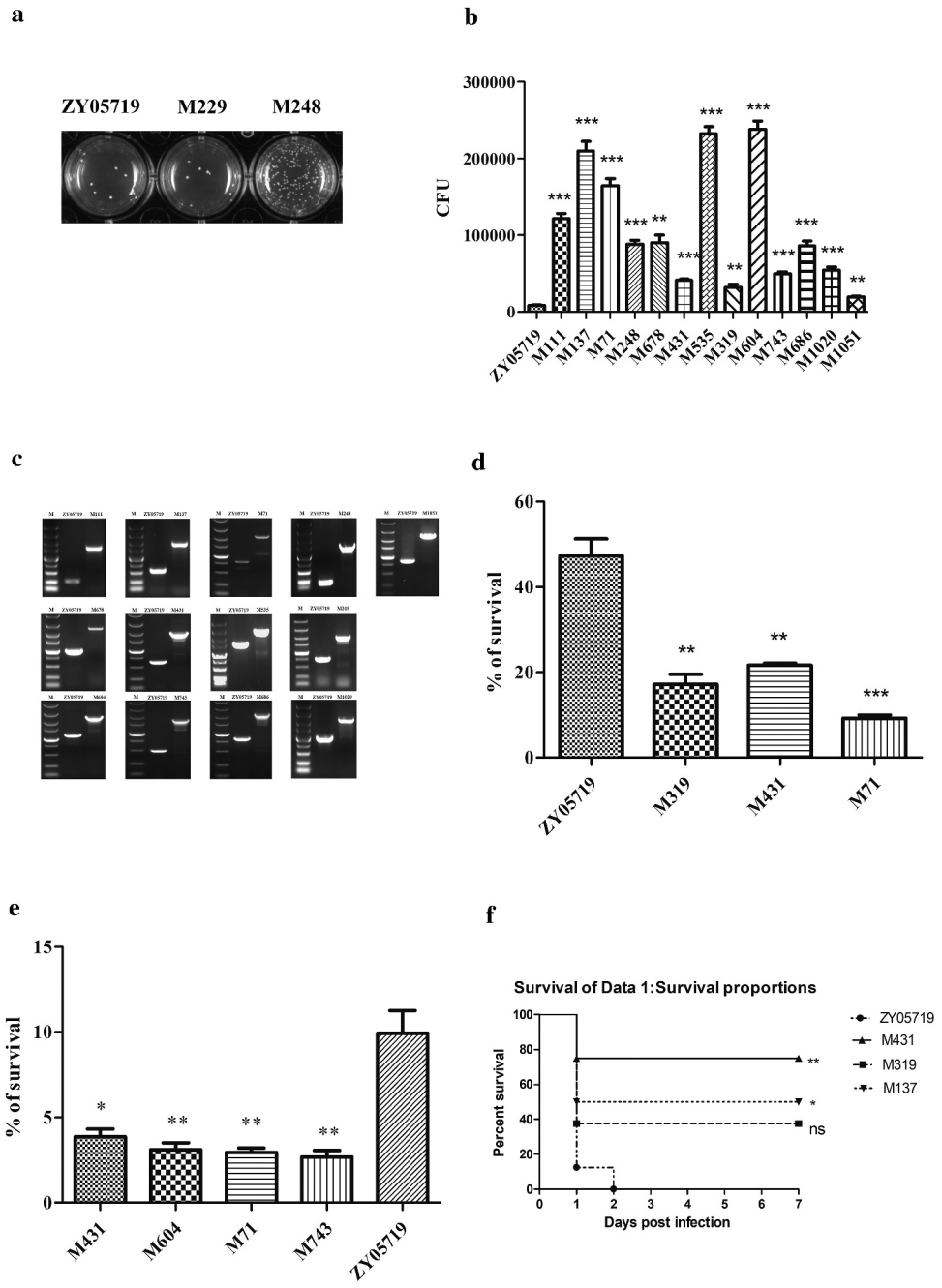

Figure 1. Screening of phagocytosis resistance transposon mutants. (a) Semiquantitative phagocytosis assay was performed for the initial screening of mutants with decreased anti-phagocytosis ability. Part of the results were presented. (b) A total of 3.0×10^5 Raw264.7 cells were infected individually with the transposon mutant strains or ZY05719 at an MOI of 10:1 for 1 h. CFUs of phagocytosed mutant strains and ZY05719 recovered from macrophages were determined (c) Confirmation of transposon insertion site. The transposon inserted gene had a 1300 bp size shift compared with the normal gene, which corresponds to the transposon sequence length. Lane M indicated the 5000 bp DNA marker. (d) Three SS2 ZY05719 transposon mutants with decreased anti-phagocytosis ability (M319, M431 and M71) were selected randomly and examined for their survival rate in whole swine blood. The survival ability of the 3 mutants decreased significantly compared to the wild-type strain. (e) Four SS2 ZY05719 transposon mutants with decreased anti-phagocytosis ability (M431, M604, M71 and M743) were selected randomly, and the survival ability in Raw264.7 cells were evaluated. The mutants showed decreased survival in Raw264.7 cells compared to the wild-type strain. Data represent the means with standard error of the mean of three samples. The statistical significance was determined by Student's *t* test. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. (f) Virulence to Balb/c mice were determined. Three mutants M431, M319, M137 and wild-type ZY05719 were injected via intraperitoneal at a dose of 3×10^8 per mouse. Each group contained 8 mice. The mutants showed attenuated virulence to different extents compared with ZY05719.

Table 3. Identification of transposon inserted genes of mutants with decreased anti-phagocytosis ability.

Mutant	gene	putative protein
M111	ZY05719_02410	ribose 5-phosphate isomerase, rpiA
M137	ZY05719_03765	branched-chain amino acid ABC transport substrate binding protein
M71	ZY05719_06345	S-transferase, GST
M248	ZY05719_05150	ribose-phosphate pyrophosphokinase, prs
M678	ZY05719_03375	Type I restriction-modification system, DNA methylase subunit, HsdM
M431	ZY05719_07905	response regulator transcription factor, WalR
M535	ZY05719_03620	ABC transporter permease/hypothetical protein with unknown function
M319	ZY05719_08420	MetQ/NlpA family ABC transporter substrate-binding protein
M604	ZY05719_02775	glycosyltransferase family 1 protein, Cps2F
M743	ZY05719_05530	GNAT family N-acetyltransferase
M686	ZY05719_06620	SAM-dependent methyltransferase TehB
M1020	ZY05719_02715	glutamate 5-kinase, proB
M1051	ZY05719_01510	heat shock protein DnaK

and hence a second PCR was conducted to determine the exact location of the transposon in each sample. The primers used are listed in Table 2. All the mutants and ZY05719 were tested with primers specific to the predicted genes, and a 1300 bp size shift was evident, which represented the transposon sequence between the corresponding band of the wild-type strain (Figure 1(c)). The results demonstrated that the transposons were inserted within the coding regions of the identified genes.

Survival ability in macrophages and whole blood was attenuated in mutant strains

The whole blood contained neutrophils and other polymorphonuclear leukocytes (PMNs) that would also engulf pathogens. Examining the survival ability in whole blood could also indicate phagocytosis resistance to some extent. We discovered that the survival rate of M319, M431, and M71 was significantly reduced compared with the wild-type strain (Figure 1(d)), suggesting the survival abilities of the mutants in whole blood were significantly attenuated.

The phagocytosis assay method in our experiment was based on the CFU of surviving bacteria that were engulfed by macrophages. Macrophages could not only internalize the bacteria, but could also eliminate the engulfed bacteria. Therefore, we also compared the survival ability of mutant strains M431, M604, M71, M743, and ZY05719 in the Raw264.7 cells in this assay, and observed that the intracellular survival ability of the mutant strains decreased significantly compared to ZY05719 (Figure 1(e)).

Transposon mutants with decreased phagocytic resistance impaired the virulence of SS2 in vivo

The process of phagocytosis is an important step in innate immunity. The mutants with attenuated phagocytosis resistance might be more vulnerable in the host. To evaluate the virulence of the strains with reduced phagocytosis resistance ability, we infected Balb/c mice at a dose of 3×10^8 CFU per mouse. In our experiment, we observed that transposon mutant M137-challenged mice showed a 50% survival rate, M431-infected mice showed a 75% survival rate, M319-infected mice showed 37.5% survival rate, while the wild-type ZY05719-challenged mice showed a 0% survival rate (Figure 1(f)). The results suggested that the transposon mutants we randomly selected had attenuated virulence compared with the wild-type strain in the mouse infection model to a different extent.

Phagocytic and adhesion to macrophages, and virulence of knockout strains in vivo

From the evaluation of the characteristics of the mutant strains and their previously reported functions, we focused on the *MetQ* and *WalR* genes for further research. The anti-phagocytosis phenotype of Δ*MetQ*, Δ*WalR*, and their corresponding complementary strains CΔ*MetQ* and CΔ*WalR* were examined. The phagocytosis resistance abilities of Δ*MetQ* and Δ*WalR* decreased, and were in accordance with those of M319 and M431, respectively. Phagocytic resistances of CΔ*MetQ* and CΔ*WalR* complementation strains were restored to the level of the wild-type strain (Figure 2(a,b)). Since the phagocytic rates of the wild-type strain and knockout strains were relatively low, M604, which was a capsular polysaccharide (CPS) negative mutant with high phagocytic rate was chosen as a positive control for the phagocytosis assay.

The adhesion to macrophages was examined in Δ*MetQ*, Δ*WalR*, CΔ*MetQ,* and CΔ*WalR*, and it showed that only the adhesion rates of Δ*WalR* varied significantly among the strains (Figure 2(c)).

To validate the virulence results of transposon mutant strains, two knockout strains Δ*MetQ,* and Δ*WalR*, as well as the corresponding complemented strains CΔ*MetQ* and CΔ*WalR* were compared with wild-type strain ZY05719 for their virulence in Balb/c mice. Measures of their virulence were similar to the corresponding transposon mutants M431 and M319, in which the Δ*WalR* showed 75% survival rate, Δ*MetQ*-infected mice showed 37.5% survival, wild-type showed 0% survival rate. CΔ*WalR*-challenged mice showed 12.5% survival, and CΔ*MetQ*-challenged mice showed 25% survival (Figure 2(d)).

Growth characteristics of mutant strains

To determine whether the growth kinetics of mutants contribute to the decreased anti-phagocytic ability and virulence, the growth characteristics of transposon mutants were measured in THY. Results showed that the growth of

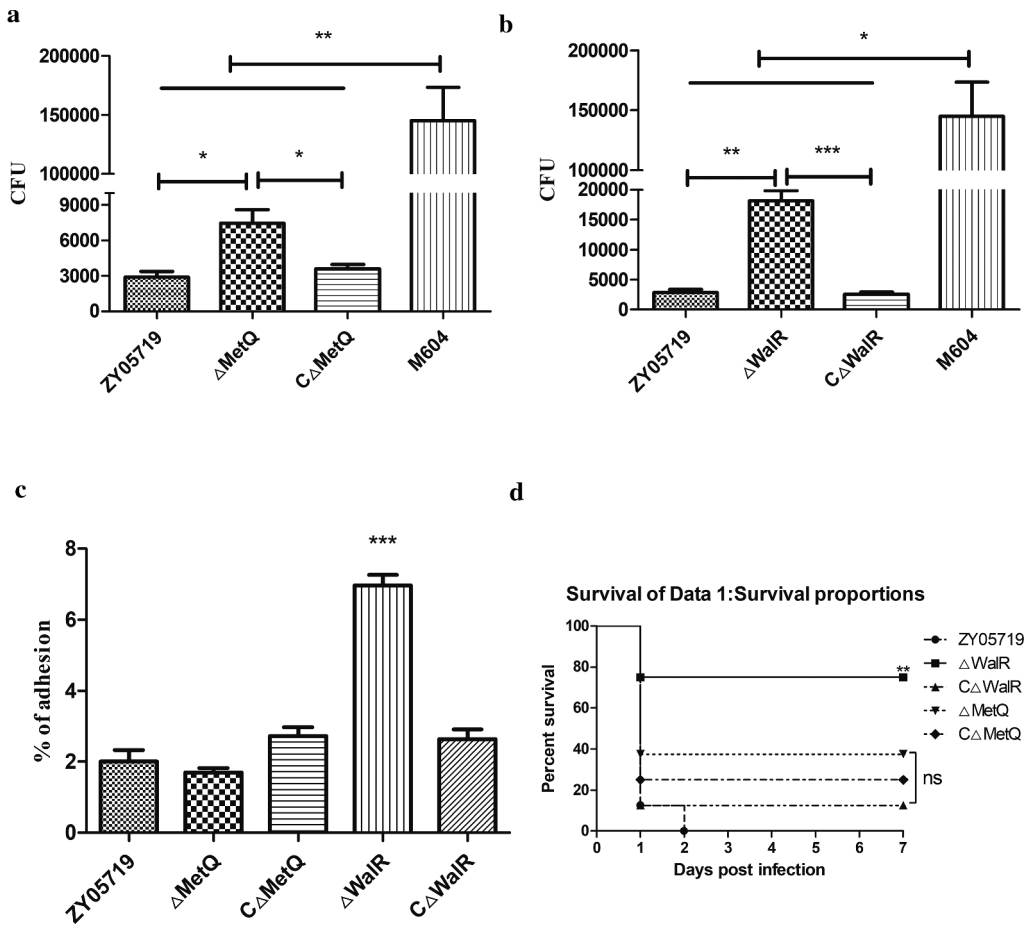

Figure 2. Phagocytosis, adhesion rate and virulence of knockout strains Δ*MetQ* and Δ*WalR*. (a) A total of 3.0 × 10^5 Raw264.7 cells were infected separately with the Δ*MetQ*, CΔ*MetQ* or ZY05719 at an MOI of 10:1 for 1 h. CFUs of phagocytosed bacteria recovered from macrophages were determined. Δ*MetQ* showed M319 similar impairment in phagocytosis resistance. CΔ*MetQ* restored the anti-phagocytosis ability to some extent. (b) A total of 3.0 × 10^5 Raw264.7 cells were infected separately with the Δ*WalR*, CΔ*WalR* or ZY05719 at an MOI of 10:1 for 1 h. CFUs of phagocytosed bacteria recovered from macrophages were examined. Δ*WalR* were more susceptible to phagocytosis, whereas CΔ*Wal*R recovered the phenotype to the level of ZY05719. (c) Raw264.7 cells were pretreated with 1 μg/mL cytochalasin D for 30 min at 37°C under 5% CO_2. Cells were infected with bacteria (Δ*MetQ*, Δ*WalR*, CΔ*MetQ*, CΔ*WalR* or wild-type ZY05719) at an MOI of 10:1 for 2 h. Adhesion to macrophages were examined. Except for Δ*WalR*, adhesion rates were not significantly different from that of ZY05719. Data are shown as the means and standard error of the mean from three independent experiments. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. (d) Balb/c mice were challenged with Δ*MetQ*, Δ*WalR*, CΔ*MetQ*, CΔ*WalR* or wild-type ZY05719 via intraperitoneal injection at a dose of 3 × 10^8 per mouse. Each group contained 8 mice. Virulence of Δ*MetQ* and Δ*WalR* were evaluated and the virulence to Balb/c mice were attenuated to different extent compared with ZY05719. The results were similar with the virulence of the corresponding transposon mutant strains M319 and M431. Complemented strains recovered the phenotype to the level of wild-type ZY05719. $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

the mutant strains did not have obvious differences compared with wild-type ZY05719 (Figure 3(a)). Hence, the growth kinetics did not contribute to the changes in phagocytic resistance and pathogenesis of the mutants.

Transmission electron microscopy (TEM) observation of capsular polysaccharide (CPS)

The SS2 capsule is a well-known anti-phagocytic factor. The capsule was examined in mutant stains to determine if capsule changes resulted in decreased phagocytic resistance ability. TEM was used to observe the capsule structures of two mutant strains, M431 and M604, and the wild-type ZY05719. M604 was the *Cps2F* insertional mutant strain. The results showed that the capsule of M604 could hardly be observed. However, the CPS thickness of M431 showed no significant changes compared with that of the wild-type strain (Figure 3(b)). The CPS thickness was also measured using Image J software and shown in histogram (Figure 3(b)). Therefore, factors other than CPS contributed to the anti-phagocytic ability of SS2 and these will require further study.

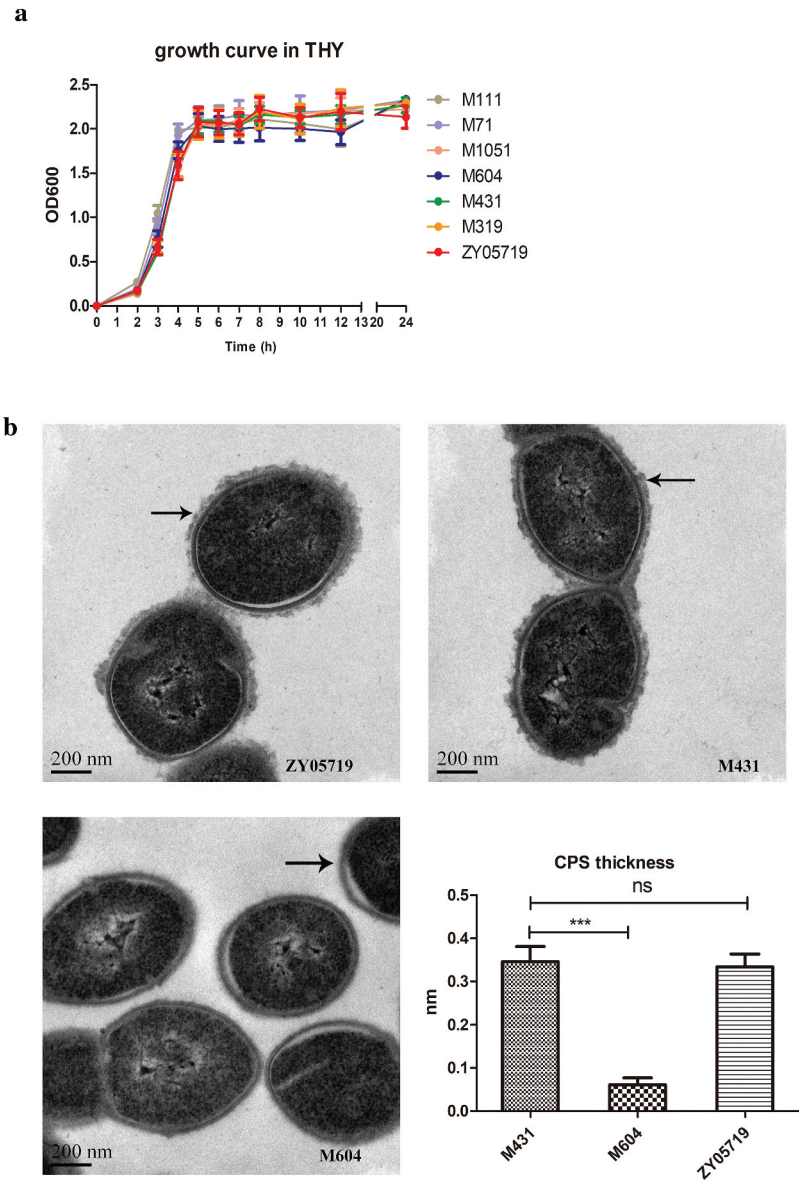

Figure 3. Growth kinetics in THY and CPS biosynthesis. (a) A subset of the 13 mutant strains were examined for their growth characteristics in THY at 37°C with vigorous shaking. There were no obvious differences in growth characteristics among mutants and wild-type ZY05719. Data are shown as the means with standard deviations from three independent experiments. (b) TEM images show the capsule of M431, M604 and ZY05719. Black arrows indicate the CPS of SS2, and the scale bars are 200 nm. CPS thickness of M431 and ZY05719 did not change obviously. CPS of M604 could hardly be visualized. The capsule thicknesses were quantified and shown in histogram. Data are shown as the means and standard error of the mean. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Peptidoglycan biosynthesis-related gene expression in M431

M431 is the *WalR* insertional mutant strain, and *WalR* was reported to be associated with cell wall hemeostasis [16]. We examined transcription of the peptidoglycan biosynthesis-related genes *PcsB, HP1065*, two *LysM* genes (known to encode the peptidoglycan-binding domain proteins), *LytB, murA, murG, pbp1a, pbp1b,* and *pgdA*. Since M431 is the *WalR* insertional mutant, we also examined the transcription of *WalR*, and the mRNA level of *WalR* was decreased 0.67-fold relative to wild-type ZY05719 ($P < 0.0001$); these results showed that M431 is a *WalR* knock-down strain. M431 was more sensitive to phagocytosis compared with ZY05719 in the previously described experiment. In our experiment, we discovered that the *LysM* peptidoglycan-binding domain protein *LysM2173* (ZY05719_10260) increased approximately

1.8-fold ($P < 0.001$), *HP1065* (ZY05719_01065, hypothetical protein 1065) decreased significantly ($P < 0.001$), *pbp1a* (penicillin-binding protein) decreased 0.72-fold compared to ZY05719 ($P < 0.05$) (Figure 4(a)). We speculated that *WalR* negatively regulates *LysM2173* and activates *HP1065* and *pbp1a* in SS2, which would thereby affect the phagocytosis resistance of SS2.

Knockout of *HP1065* impaired phagocytosis resistance of SS2.

We discovered that *LysM2173* transcription increased while that of *HP1065* decreased in the *WalR* mutant strain, which led us to speculate that overexpression of *LysM2173* and deletion of *HP1065* might impair the phagocytosis resistance of SS2. The knockout strain of *HP1065* (Δ*HP1065*) significantly attenuated the phagocytosis resistance ability of SS2. However, we constructed a *LysM2173* overexpression strain, C*LysM*, and the phagocytosis rate of C*LysM* did not change significantly compared with that of the wild-type strain (Figure 4(b)). Hence, *HP1065* might contribute to the phagocytosis resistance ability of SS2, M431 led to the downregulation of *HP1065* which could have resulted in the decreased anti-phagocytosis of SS2. Meanwhile, the upregulation of *LysM2173* was not involved with the phagocytosis resistance of SS2.

Discussion

Streptococcus suis is an important zoonosis pathogen that causes sudden death, acute sepsis, arthritis, and meningitis in humans and pigs; it has caused great losses in the swine industry, and posed a threat to public health. The symptoms of SS2 infection suggest the possibility that SS2 can evade innate immunity. The phagocytosis process plays an important role in innate immunity, and it is also involved in the acquired immune response by antigen presentation. Actin cytoskeleton rearrangement is the basis of the phagocytosis processes. Actin recruitment is regulated by Rho family GTPases, including RhoA, Rac, and Cdc42 [24]. Phagocytic pathways are initiated by various receptors, Arp2/3, and WASP/WAVE family proteins are triggered to further activate the actin remodeling [25]. Effective phagocytosis by macrophages also includes elimination phagocytosed bacteria by lysosome enzymes, acidic environments, ROS and RNS [26]. In response, pathogens have evolved to counteract host cell-mediated phagocytosis. Some pathogen bacteria produce proteins capable of hijacking the host proteins to impact the actin cytoskeleton. Type III secretion system effectors of enteropathogenic *Escherichia coli* (EPEC) and enterohaemorrhagic *Escherichia coli* (EHEC), such as EspH, could interfere with the cytoskeleton dynamics by binding to RhoGEF and resulted in inhibition of Rho activation and actin dynamics [27]. YopT, a type III secretion system

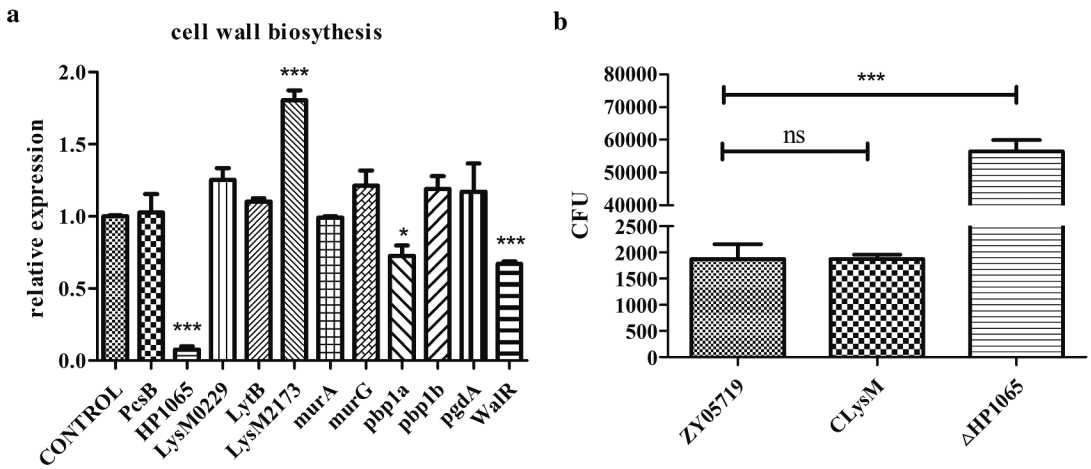

Figure 4. Peptidoglycan biosynthesis-related genes in M431. (a) Ten peptidoglycan biosynthesis-related genes were examined by qPCR for their mRNA levels in M431 and wild-type strain ZY05719. Levels of *HP1065* and *pbp1a* decreased significantly in M431 compared with those in ZY05719, while levels of *LysM2173* increased significantly. Each sample was assayed in two replicates and the data represented the means and standard error of the mean from three independent experiments. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. (b) Deletion of *HP1065* (Δ*HP1065*) and overexpression of *LysM2173* in wild-type strain ZY05719 (C*LysM*), were constructed and the phagocytosis assay was conducted. Raw264.7 cells were infected separately with the Δ*HP1065*, C*LysM* or ZY05719 at an MOI of 10:1 for 1 h. CFUs of phagocytosed bacteria recovered from macrophages were examined. Phagocytosed Δ*HP1065* increased significantly compared with ZY05719. Whereas, engulfed C*LysM* did not change significantly compared with wild-type ZY05719. Data represent the means and standard error of the mean from three independent experiments. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

effector of *Yersinia*, could cleave Rho GTPases and result in disruption of actin cytoskeleton and contribute to the anti-phagocytic ability of *Yersinia* [28]. There are various activating and inhibitory immunoreceptors in neutrophils that modulate the hemeostasis of neutrophil function. Furthermore, some pathogens have evolved to manipulate the associated pathways of neutrophils to evade the immune processes. For example, sialylated capsular polysaccharide of Group B *Streptococci* (GBS) can interact with inhibitory Siglec-9 and inhibit the phagocytosis process. β-protein of GBS can bind Siglec-5 to dampen macrophage phagocytosis [29,30]. When *Escherichia coli* bind to inhibitory FcγRIII, phagocytosis of *Escherichia coli* mediated by class A scavenger receptor MARCO is inhibited [31]. Some bacteria can counteract the host cells when engulfed by macrophages or even survive within professional phagocytes. For *Mycobacterium tuberculosis*, a membrane-associated protein Rv3671 c was involved with resistance to acidic environments [32]. The *KatB* gene of *Mycobacterium tuberculosis* has the function of catalase, peroxidase, and peroxynitritase, and was shown to be involved with the resistance of phagocyte oxidative burst [33]. There were a number of factors that contributed to the resistance to opsonophagocytosis and phagocytosis in Streptococcus. In group A Streptococci, hyaluronic acid capsule and surface M protein were famous for their anti-phagocytosis ability [34,35]. In SS2, it has been reported that a variety of factors could help escape the complement deposition and activation. CPS is the well-established anti-phagocytosis factor [9]. IdeSsuis could degrade porcine IgM to facilitate evasion of complement opsonization and killing [36]. However, mechanism of SS2 resistance to non-opsonophagocytosis are unknown and remain to be elucidated. Therefore, we screened for more pathogenesis-associated genes via a SS2 mutant library constructed previously in this lab [3].

In this study, the SS2 mutant pool was screened for genes related to phagocytosis resistance and 13 related genes were identified. Among the 13 disrupted genes, *Cps2F* (M604) is a member of the capsular saccharide biosynthesis locus of SS2. The capsule of SS2 is a well-known virulence factor and is famous for its anti-phagocytic ability in Streptococcus [37,38]. Nonencapsulated SS2 were shown to be easily phagocytosed by macrophages and to be avirulent in murine and pig models of infection [9,39]. GNAT family N-acetyltransferase (M743) and HsdM (M678) might be involved in posttranslational modifications (PTMs) of bacterial proteins. In *Salmonella typhimurium*, acetylation of the pathogenicity island 1 regulator HilD could stabilize the HilD protein and reduce its affinity for DNA-binding [40]. An amino acid mutation at 94 residue was identified in glutamate 5-kinase (*proB*) (M1020) in a small colony variant (SCV) of *S. pneumoniae* [41]. The bacterial genome contains a number of ABC transporters that are involved in nutrient uptake, antibiotic resistance, protein excretion, and other functions [42]. In our screening, we identified genes related to the phagocytosis resistance of SS2; three ABC transporter components were identified, including methionine ABC transporter substrate-binding protein *MetQ* (M319), a branched-chain amino acid ABC transporter substrate-binding protein (M137) and an unknown ABC transporter permease (M535). Many ABC transporters are associated with the full virulence of pathogenic bacteria [43,44]. In our experiments, mice infected with M319 and M137 had an increased survival rate relative to wild-type. *MetQ* is the substrate-binding protein of the methionine ABC transporter, which is responsible for methionine uptake from the environment. *Neisseria gonorrhoeae MetQ* is associated with survival in macrophages and monocytes as well as the ability to adhere to epithelial cells [45]. Through *in vivo*-induced antigen technology (IVIAT), *MetQ* expression was shown to increase during *Streptococcus suis* infection, indicating that *MetQ* might be a potential virulence factor, and could facilitate an important pathogenesis mechanism of SS2 [46]. In *Streptococcus pneumoniae*, the branched-chain amino acid ABC transporter livJHMGF was necessary for its full virulence [47]. *MetQ* and *livM* (branched-chain amino acid ABC permease) have been reported to be essential for replication in cerebrospinal fluid (CSF) in *Streptococcus pneumoniae* [48]. In this study, however, except for *Cps2F* gene, the other 12 genes have not reported for their anti-phagocytosis ability.

Knockout strains Δ*MetQ* and Δ*WalR*, as well as corresponding complementary strains CΔ*MetQ* and CΔ*WalR*, were examined for their phagocytosis resistance ability and attachment ability to macrophages. Rates of phagocytosis increased in the knockout strains and were restored to wild-type levels in the complementary strains. However, the adhesion rates of the knockout strains and complementary strains did not correlate with the phagocytic rates, indicating that attachment to macrophages might not contribute to the phagocytosis of SS2; this finding was in agreement with a previous report [49]. Growth kinetics in THY were determined for the transposon mutants, and there were no significant differences in growth characteristics among the mutant strains and wild-type strain, indicating the phagocytosis resistance of SS2 was not metabolic dependent process. The capsule of SS2 is well-known for its anti-phagocytic ability. Therefore, the capsules of two mutant strains M431 and M604, and wild-type ZY05719 were examined by TEM and we discovered no obvious differences in capsule thickness between M431 and ZY05719. M604 is a *Cps2F* insertional mutant, and CPS

could hardly be detected in M604. The result indicated that capsule of SS2 was not the only reason for its anti-phagocytosis ability.

M431 is a *WalR* insertional mutant, and *WalR* is part of the two-component signal transduction system *Wal*RK (*YycFG*), which was reported to be related to peptidoglycan formation and cell wall hemeostasis. In *S. aureus, YycFG* regulates cell wall metabolism, including autolysins (*atlA*), transglycosylase (*IsaA, SceD*), and CHAP amidase domain-containing proteins [15,50]. In *Streptococcus pneumoniae*, the *Wal*RK system is essential for growth and plays an important role in virulence and competence [51]. *Wal*RK of *S. pneumoniae* could activate the *PcsB, pspA, lytB,* and *LysM* repeat proteins [52]. *WalRK* could also indirectly regulate fatty acid biosynthesis in *S. pneumoniae* [53]. In *Streptococcus mutans, WalR* directly regulates *comCDE, nlmC,* and *copY* [54], which are involved in competence and biofilm formation. *S. mutans WalKR* could also regulate *atlA* and autolysis [55]. In our experiment, the cell wall homeostasis-associated genes were examined, and we observed that the *HP1065* and *pbp1a* transcripts were significantly reduced in M431. However, one of the peptidoglycan-binding proteins, *LysM2173*, was negatively regulated by *WalR*, which was in contrast with a previous report [52,56]. The knockout strain Δ*HP1065* was more sensitive to phagocytosis, indicating that the disruption of the *HP1065* gene might result in the attenuated phagocytosis resistance in M431. HP1065 showed 29% identity to choline-binding surface protein A (PspA) of *S. pneumoniae*. PspA is a virulence factor in *S. pneumoniae* that are involved in the evasion of the complement system [57]. PspA could also contribute to the resistance of killing by neutrophil extracellular traps (NETs) [58]. It was also suggested that *LysM* contributed to the phagocytic resistance of SS2. *LysM* deletion strain were more easily ingested by macrophages [59]. In our experiment, the transcription of *LysM* increased in M431. However, overexpression of *LysM* in ZY05719 did not impair the phagocytosis resistant ability of SS2 in our experiment.

In summary, we screened approximately 1100 mutants from the SS2 mutant library constructed by our lab for phagocytic resistance-related genes and identified 13 gene disruptions that led to increased susceptibility to phagocytosis by macrophages. Randomly selected mutants from these 13 mutants showed decreased survival ability in whole blood and attenuated virulence in mice, indicating that anti-phagocytosis of SS2 was related to the pathogenesis of SS2. The *WalR* mutant (M431) downregulated the transcription of *HP1065*. Δ*HP1065* showed attenuated phagocytosis resistance relative to the wild-type strain, indicating *WalR* might regulate the expression of *HP1065* and thereby contribute to the phagocytic resistance. Even though most of the screened genes have been suggested to be associated with the phagocytosis resistance of SS2, the mechanism of their functions is not well understood. Therefore, screening was the first step to detect more phagocytosis-related genes that were not discovered before, and further research is needed for each gene to explore its role in phagocytosis resistance and the pathogenesis mechanism.

Acknowledgments

We thank for all the staff for the help and suggestions in the experiment.

Author contributions

HF, XP and HZ conceived this experiment. XP and ML conducted the experiments. XP analyzed the data. XP fulfilled the manuscript. All authors approved the final manuscript.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This study was supported by the National Key Research and Development Program (2017YFD0500203), the National Natural Science Foundation of China (31672574, 31872480), the Primary Research & Development Plan of Jiangsu Province (BE2017341), and the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD).

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