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Identification of novel genes associated with anti-phagocytic functions in *Streptococcus equi* subsp. *zooepidemicus*

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ABSTRACT

The anti-phagocytic abilities of bacteria often affect bacterial pathogenicity. Here, random mutant library of *Streptococcus equi* subsp. *zooepidemicus* (SEZ) was constructed using transposon mutagenesis. After careful screening, 30 transposon mutants with different transposon insertion sites were identified by conducting quantitative phagocytosis and insertion-site confirmation assays, whose anti-phagocytic abilities were significantly reduced relative to the wild-type strain. Insertion sites of 19 strains were monocistronic, including genes coding membrane proteins, transporters, and enzymes with unknown pathological function, such as *sadM*, *adhP*, *purD*, *guaA*, alpha-galactosidase coding gene, ABC transporter permease coding gene, metallo-beta-lactamase coding gene, and three secreted enzyme coding genes *spuZ*, *slaB*, and *endoS*, as well as known virulence factor coding genes, such as *hasA* and *szM*. The insertion sites of another 11 strains were polycistronic. We focused on four monocistronic-mutant strains: M*htpZ*, M*spuZ*, M*slaB*, and M*endoS*. The anti-phagocytic abilities of not only the mutants that were precoincubated with the recombinant proteins, but also the complement strains were significantly more pronounced than those of all four corresponding mutants. The polyclonal antiserum against SlaB or EndoS also significantly decreased the anti-phagocytic capacity of wild-type SEZ. All four mutants exhibited significantly decreased viability in whole blood and reduced lethality in mice relative to the wild-type strain. Thus, we identified a variety of new anti-phagocytic factors, particularly multiple SEZ secreted enzymes. These factors are instrumental in the phagocytic resistance of SEZ in the absence of opsonin. Our results provide a framework for further studies of SEZ pathogenesis and relevant vaccine development for novel potential targets.

1. Introduction

Streptococcus equi subsp. *zooepidemicus* (SEZ), a Lancefield Group C *Streptococcus*, is an important opportunistic pathogen that primarily affects the equine and swine industries, leading to serious animal welfare problems and severe economic losses (Feng, 1977; Bjornsdottir et al., 2017). SEZ has no obvious host restrictions, and may infect cattle, goats, dogs, cats, monkeys, chickens, and other animals (Acke et al., 2015; Bisgaard et al., 2012). SEZ may also sometimes act as zoonotic pathogen, infecting humans who have close contact with infected animals or animal products (Hoyer-Nielsen et al., 2018). SEZ generally infects the mucous membranes and skin, causing diseases such as sepsis, arthritis, meningitis, and endocarditis in pigs and humans (Hoyer-Nielsen et al., 2018; Soedarmanto et al., 1996). Despite the clinical relevance of SEZ, the factors that contribute to its virulence are not well known.

The internalization and subsequent destruction of pathogenic microorganisms by phagocytic cells, including macrophages, are important aspects of innate immunity, and they promote antigen presentation to initiate the adaptive immune response (Flannagan et al., 2009). The anti-phagocytic features of pathogenic microorganisms contribute to their colonization, infection, and dissemination in the host (Flannagan et al., 2009). Through continuous co-evolution, host phagocytic cells have developed a variety of recognition, engulfment, and elimination methods, while pathogenic bacteria have developed corresponding resistance and escape methods (Flannagan et al., 2009). As

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SEZ is typically an extracellular bacterial pathogen, this bacterium is susceptible to direct contact and phagocytosis by host phagocytic cells (Xu et al., 2016). SEZ mutants with significantly reduced anti-phagocytic abilities are often significantly less pathogenic to the host, as compared to wild-type strains (Fan et al., 2009; Ma et al., 2012; Wei et al., 2012).

Some SEZ virulence factors have been reported to be associated with anti-phagocytic activities. The SEZ capsular polysaccharide allows the bacterium to resist internalization by phagocytes (Wibawan et al., 1999). The deficiency of phagocyte surface molecule CD44, which binds to the SEZ hyaluronic acid capsular, significantly reduced the phagocytic capacity of macrophage to SEZ, indicating that the recognition of capsule is implicated in phagocytosis activation (Fu et al., 2017). In addition to the capsule, two M-like proteins, Szp and SzM, also increase the phagocytosis resistance of SEZ by reducing the deposition of the C3 complement on the bacterial surface (Boschwitz and Timoney, 1994; Ma et al., 2012; Wei et al., 2012). IdeZ, a SEZ cysteine proteinase, also inhibits IgG-mediated complement deposition and phagocytosis by degrading IgG (Greta et al., 2009). Previously, we found that ENuc and 5Nuc, two extracellular SEZ nucleases, inhibit the phagocytosis of SEZ by neutrophil by degrading neutrophil extracellular traps (NETs) DNA (Ma et al., 2017). However, previous studies of SEZ have primarily focused on opsonic phagocytosis. The anti-phagocytic mechanisms used by SEZ in the absence of opsonin remain unclear. In this work, we aim to investigate these mechanisms.

2. Materials and methods

2.1. Strains, plasmids, and cell lines

The bacterial strains and plasmids used in this study are listed in Table 1. SEZ virulent strain ATCC35246 was purchased from the American Type Culture Collection (ATCC). The virulent SEZ strain CY was isolated in Jiangsu province, China by the Jiangsu Academy of Agricultural Sciences and maintained in our laboratory. The commercial SEZ avirulent vaccine strain ST_{171} was generated from a virulent strain which isolated from diseased pigs in Guangdong province, China. The virulent *Streptococcus equi* subsp. *equi* (SEE) strain CVCC573 and the virulent *Streptococcus pyogenes* strain CVCC593 were purchased from the China Veterinary Culture Collection Center (CVCC). The virulent *Streptococcus suis* serotype 2 strain ZY05719 was collected and maintained by our laboratory. The virulent *S. suis* serotype 2 strain P1/7 was purchased from the ATCC. All *Streptococcus* strains were cultured in Todd-Hewitt (TH) medium at 37 °C. *Escherichia coli* strains DH5α and BL21, which were cultured at 37 °C in Luria-Bertani medium, were used as hosts for the insertion mutation plasmid pMar4s (Liu et al., 2016), the prokaryotic expression plasmid pET28a, and the *E. coli-Streptococcus* shuttle plasmid pSET2 (Takamatsu et al., 2001). To construct the recombinant plasmids and select mutants, spectinomycin was added to the medium at 100 μg mL^{-1} for SEZ and 50 μg mL^{-1} for *E. coli*, and kanamycin was added to the medium at 50 μg mL^{-1} for both SEZ and *E. coli*. The mouse macrophage cell line RAW264.7, which was immortalized with Abelson murine leukemia virus (https://www.atcc.org/products/all/TIB-71.aspx), was purchased from ATCC and cultured in Dulbecco's modified Eagle's medium (DMEM) high-glucose, supplemented with 10% fetal bovine serum (FBS), at 37 °C under 5% CO_2.

2.2. Construction of the SEZ transposon mutant library

Preparation of competent bacteria and electrotransformation of SEZ were performed as previously reported (McLaughlin and Ferretti, 1995), with some modifications. In brief, the strain ATCC35246 was cultured in TH medium supplemented with 20 mg mL^{-1} yeast extract, 40 mM DL-threonine, and 100 μg/mL hyaluronidase at 37 °C with shaking at 180 rpm until the OD_{600} of culture medium was 0.5–0.6. The culture medium was then placed on ice for 30 min. After cooling, the

Table 1
Bacterial strains and plasmids used in this study.

Names	Notable characteristic(s)[a]	Source or reference
Bacterial strains		
DH5α	Host for plasmid cloning	Purchased from Tiangen
BL21	Host for recombinant protein expression	Purchased from Tiangen
ATCC35246	Virulent strain of SEZ isolated from an infected pig in Sichuan province, China	Purchased from ATCC
CY	Virulent strain of SEZ isolated from an infected pig in Jiangsu province, China	Lab collection
ST_{171}	Commercial avirulent vaccine strain of SEZ generated from a virulent strain which isolated from diseased pigs in Guangdong province, China.	Lab collection
CVCC573	Virulent strain of *S. equi subsp. equi* isolated from an infected horse in Beijing, China	Purchased from CVCC
CVCC593	Virulent strain of *S. pyogenes* isolated from a human in Japan	Purchased from CVCC
ZY05719	Virulent strain of *S. suis* serotype 2 isolated from a dead pig in Sichuan province, China	Lab collection
P1/7	Virulent strain of *S. suis* serotype 2 isolated from a human in Holland	Purchased from ATCC
M*slaB*	ATCC35246 transposon mutants obtained by TnYLB-1 insertion within the ORF of gene *slaB*	This study
M*endoS*	ATCC35246 transposon mutants obtained by TnYLB-1 insertion within the ORF of gene *endoS*	This study
M*spuZ*	ATCC35246 transposon mutants obtained by TnYLB-1 insertion within the ORF of gene *spuZ*	This study
M*htpZ*	ATCC35246 transposon mutants obtained by TnYLB-1 insertion within the ORF of gene *htpZ*	This study
CM*slaB*	Complemented strain of ATCC35246 M*slaB*, Spc^r	This study
CM*endoS*	Complemented strain of ATCC35246 M*endoS*, Spc^r	This study
CM*spuZ*	Complemented strain of ATCC35246 M*spuZ*, Spc^r	This study
CM*htpZ*	Complemented strain of ATCC35246 M*htpZ*, Spc^r	This study
Plasmids		
pMar4s	The *Streptococcus* random insertion mutation plasmid, Spc^r and Kan^r	Liu et al., 2016
pET28α	Prokaryotic recombinant protein expression plasmid, Kan^r	Purchased from TaKaRa
pET28α:: *slaB*	pET28α containing *slaB* in the *Eco*R I-*Sal* I restriction sites, Kan^r	This study
pET28α:: *endoS*	pET28α containing *endoS* in the *Eco*R I-*Sal* I restriction sites, Kan^r	This study
pET28α:: *spuZ*	pET28α containing *spuZ* in the *Eco*R I-*Sal* I restriction sites, Kan^r	This study
pET28α:: *htpZ*	pET28α containing *htpZ* in the *Eco*R I-*Sal* I restriction sites, Kan^r	This study
pSET2	*E. coli - Streptococcus* shuttle cloning plasmid, Spc^r	Takamatsu et al., 2001
pSET2:: *slaB*	pSET2 containing *slaB* in the *Eco*R I-*Bam*H I restriction sites, Spc^r	This study
pSET2:: *endoS*	pSET2 containing *endoS* in the *Eco*R I-*Bam*H I restriction sites, Spc^r	This study
pSET2:: *spuZ*	pSET2 containing *spuZ* in the *Eco*R I-*Bam*H I restriction sites, Spc^r	This study
pSET2:: *htpZ*	pSET2 containing *htpZ* in the *Eco*R I-*Bam*H I restriction sites, Spc^r	This study

[a] Spc, spectinomycin; Kan, kanamycin; ORF, open reading frame.

Table 2
Primers used in this study.

Names	Oligonucleotide sequence (5'–3')[a]	Products
oITR	CCCCTGCAGTAACAGGTTGGCTGATAAGTCCCCGGTCT	TnYLB-1
oIPCR1	GCTTGTAAATTCTATCATAATTG	Amplified fragments of IPCR
oIPCR2	AGGGAATCATTTGAAGGTTGG	Amplified fragments of IPCR
SLAB-F	GCGGAATTCGCAGATACTGCACCTGCAAGT	*slaB*
SLAB-R	GCCGTCGACGTTAACATCCAATAGCACCTACCGT	*slaB*
ENDOS-F	CGCGGATCCATGGTGGCTATATTAGCTGC	*endoS*
ENDOS-R	GCCGTCGACGCTAGTCTGCCTTTGATTTTTCTGA	*endoS*
SPUZ-F	TGGGTCGCGGATCCGAATTCATGTTAAATGTAAAAAAACGTCCTG	*spuZ*
SPUZ-R	CGGCCGCAAGCTTGTCGACGCTAGGCTCTTATGGTCTTACTGGTT	*spuZ*
HTPZ-F	TGGGTCGCGGATCCGAATTCAAGACAAAACAGACAGCTAAAAAAG	*htpZ*
HTPZ-R	CGGCCGCAAGCTTGTCGACGTTAACCATGGTGGTGCTCATC	*htpZ*
CSLAB-F	GCCGGATCCTGATAAAGAGATACTCCAAAGCAT	*slaB* and its promoter
CSLAB-R	GCGGTCGACACTATCTAAAATATAAGAAATGGGA	*slaB* and its promoter
CENDOS-F	GCCGGATCCAGTTGATGATTTCAGCTCTTTTT	*endoS* and its promoter
CENDOS-R	GCGGTCGACTCCTTAGTAAATGATGCGTCTTG	*endoS* and its promoter
CSPUZ-F	TTGTAAAACGACGGCCAGTGAATTCCTGAGTATCAAAGTAAGCCCCG	*spuZ* and its promoter
CSPUZ-R	CCTGCAGGTCGACTCTAGAGGATCCAGATATGTCCCCCCTTAAAATG	*spuZ* and its promoter
CHTPZ-F	TTGTAAAACGACGGCCAGTGAATTCTTTATGTGAATAAACGAGATGGCTA	*htpZ* and its promoter
CHTPZ-R	CCTGCAGGTCGACTCTAGAGGATCCCTAGTCCCTCATACACGCACC	*htpZ* and its promoter

[a] Underlined portions of the primers correspond to restriction enzyme recognition sites. GTCGAC, *Sal* I; GGATCC, *Bam*H I; and GAATTC, *Eco*R I.

bacterial pellet was collected via centrifugation at 6000 × g at 4 °C. The pellet was then washed two times with precooled EB buffer (2.5 mM K_3PO_4 and 0.3 M sucrose in double-distilled water), and two times with 15% glycerol under the same centrifugation conditions. Finally, the bacterial pellet was resuspended in 15% glycerol and stored at −70 °C until use.

The pMar4s plasmid was electroporated into competent bacteria. The bacteria were incubated in TH medium with 20 mg/mL yeast extract and 0.3 M sucrose at 28 °C for 4 h. Then, SEZ were plated onto TH agars containing kanamycin, and cultured at 37 °C until colonies grew. Single colonies were selected and transferred onto TH agars containing either kanamycin or spectinomycin. Spectinomycin-sensitive but kanamycin-resistant colonies were retained as insertion mutants. Partial mutants were randomly selected, and the transposon TnYLB-1 was identified in the genome after DNA extraction and PCR amplification using the oITR primer (Table 2).

2.3. Identification of mutant genes in transposon mutants

The genomic DNA of each mutant was extracted using the TaKaRa MiniBEST Bacteria Genomic DNA Extraction Kit, following the manufacturer's instructions. We then digested 2 μg of genomic DNA with *Taq* I at 65 °C for 5 h. The digested DNA fragments from each mutant were recovered using the TaKaRa MiniBEST DNA Fragment Purification Kit, following the manufacturer's instructions. The DNA fragments were self-cyclized by ligation with T4 DNA ligase at 16 °C overnight. Using the ligation product as the template, and oIPCR1 and oIPCR2 as the primers (Table 2), the upstream and downstream sequences of the insertion site of the transposon, as well as partial sequences at both ends of the transposon sequence, were amplified together by PCR. The amplified products were run on 1% agarose gels, and then extracted and purified using OMEGA Gel Extraction Kits, following the manufacturer's instructions. The recovered DNA fragments were sequenced, and then aligned with BLAST to identify the insertion site of the transposon in each mutant.

2.4. Construction of complementation strains

The genomic DNA of wild-type SEZ was used as the template for PCR. The DNA fragment containing the whole open reading frame (ORF) of the inserted mutant gene and its promoter sequence was PCR amplified and cloned into the pSET2 plasmid. The verified recombinant plasmid was electroporated into the corresponding insertion mutant to construct the complementation strain. The primers used are listed in Table 2.

2.5. Anti-phagocytosis assays

Assays were performed as described previously (Redlich et al., 2012), with some modifications. Briefly, ~5.0×10^5 RAW264.7 cells cultured in each well of the 24-well plates were used for assays. The wild-type strain and the transposon mutants were cultured to an OD_{600} of 0.6 in TH broth at 37 °C, then collected using centrifugation, washed with sterile 0.1 M PBS (pH 7.4), and resuspended in DMEM. Bacteria were added to cells at a multiplicity of infection (MOI) of 10. After 2 h of incubation at 37 °C under 5% CO_2, the cells were washed three times with PBS. DMEM containing 200 μg mL^{-1} gentamicin and 10 μg mL^{-1} penicillin, which can completely kill ~5.0×10^6 CFUs of wild-type ATCC35246 within 1 h (Table S1), was added to the washed cells, and incubated for 1 h at 37 °C under 5% CO_2 to kill extracellular bacteria. Cells were washed three times with PBS, and then fully lysed by adding 1 mL of sterilized ultrapure water to each well, and incubating plates at room temperature for 10 min. The bacterial and cellular-residue suspensions were then thoroughly pipetted, diluted, and coated on TH agar plates. When the colonies of an insertion mutant significantly outnumbered those of the wild-type strain ($p < 0.05$), the anti-phagocytic activity of the mutant was considered significantly less than that of the wild-type strain. Each assay was performed three times.

2.6. Bacterial survival in macrophage

Intracellular survival analysis in RAW264.7 was performed based on previous report (Cybulski et al., 2008) with some modifications. Briefly, the protocols were the same as the anti-phagocytosis assays except that the incubation time before the cells were added to DMEM containing antibiotics was 1 h. And after 1 h of incubation with DMEM containing 200 μg mL^{-1} gentamicin and 10 μg mL^{-1} penicillin, the cellular culture was replaced with DMEM containing 20 μg mL^{-1} gentamicin and 10 μg mL^{-1} penicillin for additional 1 h. Intercellular bacteria were recovered after 1 h or 2 h of incubation with DMEM containing antibiotics and calculated using the plate count method. The survival rate was calculated as CFU2h/CFU1h × 100%. Each assay was performed three times.

2.7. Determination of the median lethal dose of different strains in mice

The median lethal dose (LD_{50}) of different strains of SEZ in mice was determined following previous studies (Fan et al., 2009), with some modifications. Briefly, every five four-week-old female specific pathogen free (SPF) class ICR mice were randomly assigned and kept in the individual cages for 72 h before infection to adapt new environment and reduce stress. SEZ was cultured in TH broth to an OD_{600} of about 0.6, collected by centrifugation, washed with sterile 0.1 M PBS (pH 7.4), and resuspended in sterile 0.1 M PBS (pH 7.4). The bacterial concentration was adjusted to 1×10^9 CFUs/mL, and then serially 10-fold diluted to the minimum concentration (1×10^2 CFUs/mL). Eight doses and 0.1 mL of each dose of the bacterial suspension were intraperitoneally injected into ICR mice. A group of five mice were injected with the same concentration of suspension. After injection, the animals were observed continuously for seven days, and survival rates were recorded. Finally, the LD_{50} was calculated according to the Bliss method (Lieberman, 1983).

2.8. Expression and purification of recombinant proteins, and preparation of antiserum

The primers used in this experiment are listed in Table 2. To express the recombinant protein, the DNA sequences of the SEZ proteins were PCR amplified, using the genomic DNA of wild-type SEZ as the template. PCR products were digested with restriction endonucleases, and ligated into the pET-28a plasmid using T4 DNA Ligase. The recombinant plasmids verified by DNA sequencing were transformed into *E. coli* BL21 (DE3). Recombinant protein expression was induced with 1 mM IPTG at 16 °C for 12 h, as soon as the OD_{600} of BL21 (DE3) growing in LB broth was 0.3. Bacterial pellets were harvested by centrifugation. Protein purification was performed using GE Healthcare nickel-chelating chromatography, following the manufacturer's instructions.

To generate the antiserum, 100 μg of purified recombinant protein in 0.1 M PBS (pH 7.4) was emulsified with an equal volume of Freund's complete adjuvant (FCA), and injected subcutaneously into rabbits at multiple sites. After 2 weeks, the rabbits were injected subcutaneously with the same amount of protein emulsified with Freund's incomplete adjuvant (FIA). All the serum samples were collected ten days after the administration of the second immunization. Rabbit blood collected from the ear veins one week prior to immunization was used to generate the negative serum.

2.9. Capsule measurements

Relative SEZ capsule production was quantified as previously described (Xu et al., 2016). Briefly, bacteria were cultured to an OD600 of about 0.5 in TH broth. Each bacterial pellet from 4 mL of broth was washed with 150 mM Tris−HCl (pH 7.0), and resuspended in 200 μL of 150 mM Tris−HCl (pH 7.0). The suspension was then mixed with 1.2 mL 12.5 mM tetraborate in 96% sulfuric acid, then incubated at 99 °C for 5 min. Next, the sample was mixed either with 20 μL of 0.5% NaOH with 0.15% m-hydroxydiphenyl, or with 20 μL of 0.5% NaOH without 0.15% m-hydroxydiphenyl as a blank control for subtraction. Changes in color were measured spectrophotometrically at a wavelength of 520 nm. This experiment was performed three times, independently.

2.10. Phagocytosis inhibition and opsonic phagocytosis assays

We tested whether the purified recombinant protein inhibited macrophage phagocytosis as has been described previously (Feng et al., 2009). Briefly, 20 μg of either the purified recombinant protein or BSA was mixed with $\sim 5.0 \times 10^6$ CFUs of the corresponding insertion mutant in DMEM. Next, either mutant or wild-type ATCC35246 was added to the macrophages. The rest of the phagocytosis assay procedure was identical to the anti-phagocytosis assays described above. The opsonic phagocytosis assay was performed as described previously (Jiang et al., 2014). Briefly, $\sim 5.0 \times 10^6$ CFUs of wild-type SEZ in DMEM were mixed with either the rabbit-derived polyclonal antiserum or the negative serum at a ratio of 9:1 (v/v). All mixtures were incubated at 37 °C for 1 h, and then the bacterial suspensions were incubated with macrophages to perform anti-phagocytosis assays as described above. Each type of assay was performed three times independently.

2.11. Bacterial survival in whole blood

Bacterial survival in whole blood was determined as previously described (Wu et al., 2016). In brief, wild-type SEZ and insertion mutants cultured in TH broth to an OD_{600} of about 0.6 were collected by centrifugation. The bacterial pellets was washed with, and then resuspended in, sterilized 0.1 M PBS (pH 7.4). Meanwhile, fresh blood was collected from the venous sinus of orbit of seven four-week old female ICR mice, and anticoagulated by adding heparin sodium at a final concentration of 100 μg mL^{-1}. The blood collection from the venous sinus of orbit is fast and can be rapid hemostasis by pressing a sterile cotton swab, which has no obvious side effects and can reduce operating time. The bacterial suspension was mixed with mouse whole blood at a volume ratio of 1:10, and incubated for 3 h at 37 °C. Aliquots of these mixtures were collected before and after incubation, 10-times diluted with PBS, and coated on TH agars. Colonies were counted after overnight growth at 37 °C. The survival rate of the bacteria in whole blood was calculated. The assays were performed three times independently.

2.12. Animal welfare statement

All procedures and experiments involving animals were performed according to the protocols approved by the Institutional Animal Care and Use Committee of Nanjing Agricultural University (Nanjing, China), as well as the National Institutes of Health Guidelines (USA). Specific pathogen free (SPF) class ICR mice were purchased from the Comparative Medicine Center of Yangzhou University, and kept in the individually ventilate cages (IVC, Ehret) with SPF class mouse special feed and sterilized water at the perennial room temperature of 25 °C. New Zealand white rabbit were obtained from the Laboratory Animal Center of the Jiangsu Academy of Agricultural Science and were fed with rabbit special feed and sterilized water. The rabbit was seronegative for antibodies against SEZ. Every two rabbits used for one kind of polyclonal antiserum against a specific recombinant protein were housed in a separate room in a high-security isolation facility at the perennial room temperature of 25 °C. FCA is a commonly used immunopotentiator that can boost polyclonal antibody production in animal models such as rabbits, which has been reported as caused no obvious adverse effects on physiologic parameters and activity levels in rabbits (Halliday et al., 2004). All cages were cleaned and disinfected before and after housing the animals. The sterilized mat litter in cages was replaced every 5 days. All surgery was performed under isoflurane anesthesia, and all efforts were made to minimize suffering. All the survival mice and rabbits after experiments were anesthetized with isoflurane and euthanized by CO_2.

2.13. Statistical analysis

Data were analyzed using GraphPad Prism version 7.0. Data were presented as means ± standard deviation or standard error. Student's *t* tests were used for all pairwise comparisons. We considered $p < 0.05$ statistically significant.

Table 3
Efficiency of transposition of TnYLB-1 to ATCC35246.

Number of experiments	Number of total kanamycin resistant strains	Number of spectinomycin sensitive but kanamycin resistant strains	Number of total spectinomycin resistant strains	Transposition efficiency
First time	386	336	50	87.05%
Second time	474	383	91	80.80%
Third time	437	371	66	84.90%
Total	1297	1090	207	84.04%

3. Results

3.1. Construction of the SEZ transposon mutant library

The *Streptococcus* random insertion mutant plasmid pMar4s, which was previously constructed by our lab (Liu et al., 2016), contains genes that confer resistance to kanamycin and spectinomycin. After pMar4s was electroporated into wild-type ATCC35246, the TnYLB-1 transposon containing the kanamycin-resistance gene was integrated into the bacterial genome, while the rest of the plasmid (containing the spectinomycin-resistance gene) was degraded during bacterial culture at 37 °C. These spectinomycin-sensitive but kanamycin-resistant strains were retained as insertion mutants. After several rounds of experimentation, about 1000 strains of SEZ random insertion mutant libraries had been constructed. The overall transposition efficiency of the TnYLB-1 transposon to wild-type SEZ ATCC35246 was about 84.04% (Table 3). Several insertion mutants were randomly selected, and TnYLB-1 was PCR amplified using the oITR primer. The PCR results indicated that all mutant groups were positive, while the wild-type SEZ group was negative (data not shown). This suggested that the insertion mutant had been successfully constructed. Previous results from our lab have verified the pronounced randomness of insertion mutations and the high single insertion rate of transposon using Southern blot analysis (Liu et al., 2016). Therefore, these two characteristics of the plasmid are no longer validated in this study.

3.2. Screening of transposon mutants with reduced anti-phagocytic ability

The anti-phagocytic properties of selected *Streptococcus* species or subspecies were compared. We identified no significant differences between the anti-phagocytic abilities and intracellular survival abilities of two wild-type SEZ strains (ATCC35246 and CY) in RAW264.7 cells ($p > 0.05$, Fig. 1A and B). The numbers of CFUs recovered from RAW264.7 cells in anti-phagocytosis assays were significantly bigger when the cells infected with SEE CVCC573, *S. pyogenes* CVCC593, *S. suis* ZY05719, and *S. suis* P1/7 as compared to the wild-type ATCC35246; the numbers of CFUs recovered from the RAW264.7 cells increased 7.93-fold, 189.58-fold, 1258.45-fold, and 1007.35-fold, respectively ($p < 0.05$, Fig. 1A). Meanwhile, intracellular survival assays showed that there is no significant difference between wild-type ATCC35246 and any other tested *Streptococcus* species/subspecies in the survival abilities in RAW264.7 within the same total incubation time as anti-phagocytosis assays (Fig. 1B). These results indicated that the anti-phagocytic abilities of SEZ were significantly greater than the other tested *Streptococcus* species/subspecies.

The SEZ ATCC35246 transposon mutant library, which contained 1090 random mutants, was screened to determine the mechanisms underlying the anti-phagocytic properties of this strain in RAW264.7 cells. After three independent assays, we identified 30 mutants with significantly more CFUs recovered from RAW264.7 cells as compared to wild-type ATCC35246 ($p < 0.05$, Fig. 1A), ranging from a 1.32-fold increase (M765; $p < 0.01$ A) to a 4349.02-fold increase (M4; $p < 0.001$ A, Fig. 1A); all mutants had different transposon insertion sites. To visualize these results, we added 900 μL semi-solid TH medium to a 100 μL solution of either wild-type ATCC35246 or a mutant recovered after RAW264.7 phagocytosis. These bacteria were grown in 24-well plates. After overnight culture, colonies of mutants with increased amount of CFUs after phagocytosis (e.g., M4 and M11) were obviously more numerous than colonies of wild-type ATCC35246 or of mutants with similar amount of CFUs to wild-type ATCC35246 after phagocytosis (e.g., M2, Fig. 1C). Due to the limitation of nutrients in the medium, the colony size of M4 and M11 with a larger number of colonies was obviously smaller than that of wild-type ATCC35246 and M2 with a smaller number of colonies in the same culture time (Fig. 1C). The phagocytosis assays employed measured the number of surviving SEZ within RAW264.7 cells. Therefore, this number will reflect not only the number of being phagocytosed, but also the number of survival within these cells. So, we determined the viability of all 30 mutant strains in RAW264.7 within the same total incubation time as anti-phagocytosis assays. The results showed that, in addition to M4, M127, M206, M420, and M687, which significantly reduced the viabilities in macrophage cells, the viabilities of other mutants in macrophages were not significantly different from that of wild-type ATCC35246. These results indicated that the anti-phagocytic abilities of these 30 mutant strains significantly reduced relative to the wild-type ATCC35246.

3.3. Identification of mutant genes in transposon mutants with significantly reduced anti-phagocytosis ability relative to the wild-type ATCC35246

The 30 insertion sites that significantly reduced the anti-phagocytic abilities of the transposon mutants were identified (Table 4). The terminator of transposon TnYLB-1 was predicted with Softberry (http://www.softberry.com). When TnYLB-1 is inserted in a gene upstream of a polycistronic site, the transcript level(s) of downstream gene(s) will be strongly reduced (Ray-Soni et al., 2016). We previously characterized the transcriptional organization of the ATCC35246 genome using transcriptome sequencing and with Rockhopper 1.2.1 (http://cs.wellesley.edu/~btjaden/Rockhopper/) (full data not shown). Of the identified insertion mutant genes, the insertion sites of 19 genes were monocistronic, and those of 11 genes were polycistronic (Table 4). The genes that transcriptional levels were affected by insertion mutations in 11 polycistronic sites were summarized and listed in Table S2. The relative positions of these 30 insertion sites with the ATCC35246 genome were mapped with SnapGene 3.2.1 (http://www.snapgene.com/) (Fig. 2). This map indicated that the genes affecting the anti-phagocytic abilities of SEZ were distributed throughout the genome, and also showed that the insertion of the transposon TnYLB-1 into the SEZ genomic DNA was highly random.

3.4. Characteristics of different transposon mutants on mice mortality, growth kinetics, and capsule production

We focused on four transposon mutants with significantly reduced anti-phagocytosis abilities: M11, M45, M283, and M554. All four insertion sites were monocistronic. The four mutants were named M*htpZ*, M*spuZ*, M*slaB*, and M*endoS*, corresponding to the inserted mutant gene. The logarithmic growth phases of the four mutants were divided into eight CFU gradients, and used to challenge mice via intraperitoneal injection. After seven days, the LD_{50} levels of mice infected with M*htpZ*, M*spuZ*, M*slaB*, and M*endoS* were 10.51-fold, 24.68-fold, 24.85-fold, and 5.90-fold, respectively, greater than in mice infected with wild-type ATCC35246 (Table 5). The *htpZ*, *spuZ*, *slaB*, and *endoS* mutations led to

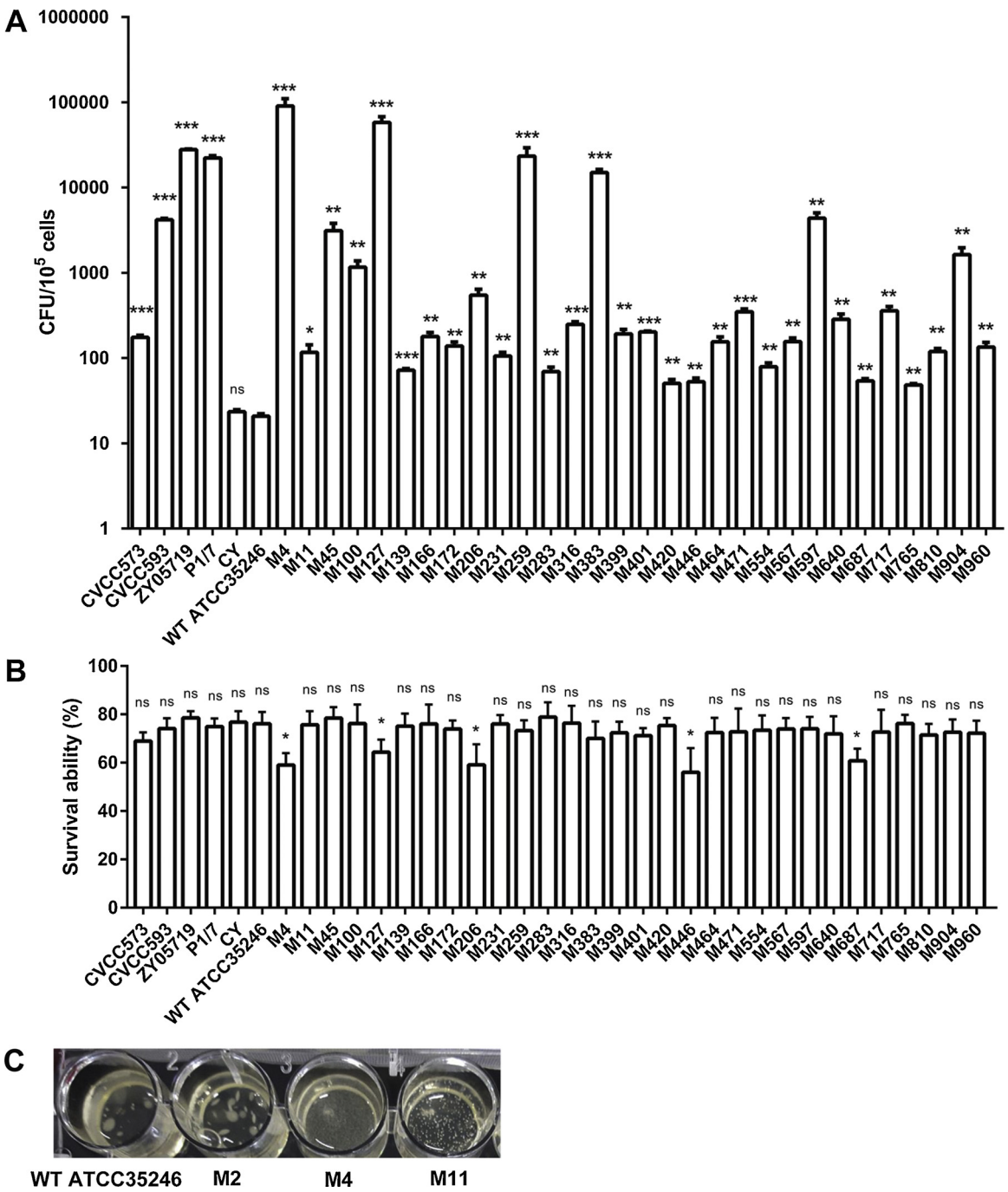

Fig. 1. Anti-phagocytosis assays and intracellular survival assays for different *Streptococcus* strains. (A) CFUs of CVCC573, CVCC593, ZY05719, P1/7, CV, wild-type ATCC35246, and ATCC35246 transposon mutants recovered after phagocytosis in different cultures of 1×10^5 RAW264.7 cells. (B) Survival rates of CVCC573, CVCC593, ZY05719, P1/7, CV, wild-type ATCC35246, and ATCC35246 transposon mutants in RAW264.7 cells calculated from intracellular survival assays. (C) Visualization of partial phagocytosis resistance screening results after wild-type ATCC35246 and its transposon mutants exposure to ~5.0×10^5 macrophages. Data shown represent mean values and standard errors of three independent experiments. Statistical significance of difference between the wild-type ATCC35246 and another strain is assessed using Student's *t* test (ns, not significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

a decrease in SEZ-associated mouse mortality, which indicated that these four genes were important for SEZ virulence in mice.

The effect of the disruption of *htpZ*, *spuZ*, *slaB* or *endoS* following transposition of TnYLB-1 on the growth of SEZ was determined. Wild-type ATCC35246, M*htpZ*, M*spuZ*, M*slaB*, and M*endoS* were cultured aerobically at 37 °C with shaking at 180 rpm in TH broth. The OD_{600} value of each culture solution was measured hourly. There was no significant difference in the growth of the four transposon mutants relative to the wild-type ATCC35246 strain (Fig. 3A). Wei et al. (2012) have shown that SEZ capsular deletion significantly reduced SEZ anti-phagocytic ability. Therefore, we also examined the capsular content of M*htpZ*, M*spuZ*, M*slaB*, M*endoS*, and wild-type ATCC35246. We found no significant differences in the capsule levels of M*htpZ*, M*spuZ*, M*slaB*, and M*endoS,* as compared to wild-type ATCC35246 (Fig. 3B). These results indicated the mutation of *htpZ*, *spuZ*, *slaB*, or *endoS* did not alter SEZ growth ability or capsular production.

Table 4
Identification of mutant genes of transposon mutants attenuated in anti-phagocytosis.

Mutant number	gene number	Transcriptional organization	Product
M4	SeseC_00231	Monocistronic	hyaluronan synthase, HasA
M11	SeseC_00825	Monocistronic	lipoprotein
M45	SeseC_01740	Monocistronic	peptidase U32 family large subunit (C1), SpuZ
M100	SeseC_00952	Monocistronic	conserved hypothetical protein
M127	SeseC_02415	Monocistronic	antiphagocytic cell surface-anchored fibrinogen-and IgG Fc-binding protein, SzM
M139	SeseC_02491	Monocistronic	S-adenosylmethionine-dependent methyltransferase
M166	SeseC_00087	Polycistronic	conserved hypothetical protein
M172	SeseC_00054	Monocistronic	alcohol dehydrogenase, AdhP
M206	SeseC_02505	Monocistronic	conserved hypothetical protein
M231	SeseC_00206	Polycistronic	alpha-galactosidase
M259	SeseC_02567	Monocistronic	membrane protein, YhgE
M283	SeseC_02520	Monocistronic	phospholipase A2, SlaB
M316	SeseC_02473	Polycistronic	fimbrial subunit protein, Fszf
M383	SeseC_01957	Polycistronic	nickel or oligopeptide import ATP-binding protein
M399	SeseC_00543	Polycistronic	membrane protein
M401	SeseC_01307	Monocistronic	GtrA-like protein
M420	SeseC_00037	Monocistronic	phosphoribosylamine-glycine ligase, PurD
M446	SeseC_02279	Monocistronic	putative stomatin/prohibitin-family membrane protease subunit
M464	SeseC_02062	Monocistronic	recombination regulator, RecX
M471	SeseC_00423	Polycistronic	sucrose operon repressor, LacI
M554	SeseC_00429	Monocistronic	endo-beta-N-acetylglucosaminidase F2 precursor, EndoS
M567	SeseC_01193	Polycistronic	ABC transporter permease protein
M597	SeseC_01793	Polycistronic	metallo-beta-lactamase superfamily protein
M640	SeseC_01867	Monocistronic	transposase
M687	SeseC_00117	Polycistronic	membrane protein
M717	SeseC_01714	Polycistronic	conserved hypothetical protein
M765	SeseC_01527	Polycistronic	membrane protein
M810	SeseC_00656	Monocistronic	conserved hypothetical protein
M904	SeseC_00169	Monocistronic	conserved hypothetical protein
M960	SeseC_01336	Monocistronic	GMP synthase, GuaA

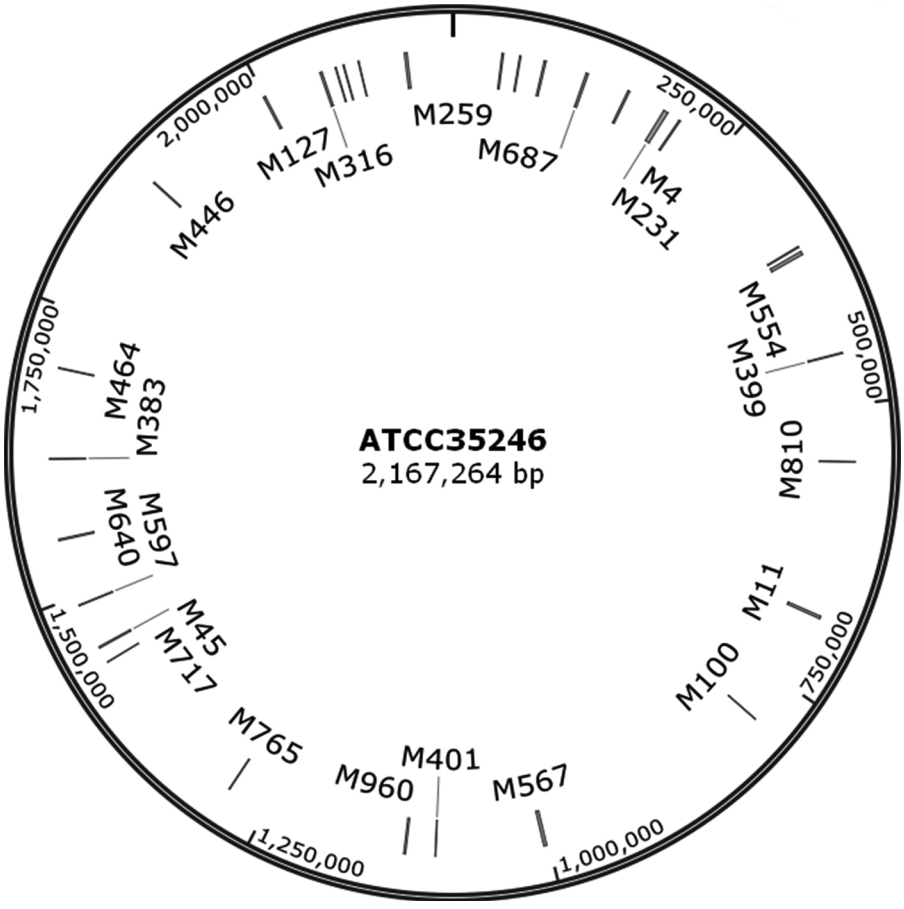

Fig. 2. ATCC35246 genome map showing the relative positions of the inserted mutant genes of the transposon mutants with significantly reduced anti-phagocytosis activities. The genes in the figure that are not marked with the relevant mutant strains are mutant genes of M139, M206, M283, M420, M172, M166, M904, M471, and M554 from downstream of the mutant gene of M316 to upstream of the mutant gene of M554 on the genome.

Table 5
Calculation of LD_{50} for SEZ in mice.

Challenge dose(CFU)	Mouse number	Death numbers					
		WT SEZ	M*htpZ*	M*spuZ*	M*slaB*	M*endoS*	ST_{171}
1×10^8	5	5	5	5	5	5	4
1×10^7	5	5	5	5	5	5	3
1×10^6	5	5	5	5	3	4	0
1×10^5	5	4	4	3	3	4	0
1×10^4	5	3	3	2	3	3	0
1×10^3	5	3	2	2	3	3	0
1×10^2	5	3	1	1	1	2	0
1×10^1	5	1	0	0	0	0	0
Value of LD_{50}		2.95×10^2	3.10×10^3	7.28×10^3	7.33×10^3	1.74×10^3	1.38×10^7

3.5. Phagocytosis inhibition by the recombinant protein, the opsonic phagocytosis of the related antiserum, and the survival ability of different strains in whole blood

The complementation strains of M*htpZ*, M*spuZ*, M*slaB*, and M*endoS* were constructed and named CM*htpZ*, CM*spuZ*, CM*slaB*, and CM*endoS*, respectively. The phagocytosis assays indicated that the levels of phagocytic activity in the RAW264.7 cells against the four complementation strains were significantly lower than the phagocytic activity levels in the RAW264.7 cells against the transposon mutants ($p < 0.05$, Fig. 4A). There was no significant difference in the anti-phagocytic abilities of CM*slaB* relative to wild-type ATCC35246. However, the anti-phagocytic abilities of CM*htpZ*, CM*endoS*, and CM*spuZ* were significantly lower than that of wild-type ATCC35246 ($p < 0.05$, Fig. 4A).

Using prokaryotic recombinant protein expression system, the recombinant HtpZ, SlaB, SpuZ, and EndoS proteins were successfully obtained and named rHtpZ, rSlaB, rSpuZ, and rEndoS, respectively (Fig. S1). rSpuZ, like other U32 family peptidases, often forms dimers (Fig. S1) (Schacherl et al., 2015). Either the corresponding recombinant protein or BSA was added to the transposon mutant in the phagocytosis assay to test phagocytosis inhibition. After the addition of rHtpZ, rSpuZ, rSlaB, or rEndoS to the corresponding transposon mutant, significantly fewer CFUs were recovered after RAW264.7 phagocytosis as compared to the mutants incubated with BSA ($p < 0.05$, Fig. 4B). The CFUs recovered from RAW264.7 phagocytosis of four transposon mutants after the addition of corresponding recombinant proteins were significantly greater than those of wild-type ATCC35246 with BSA ($p < 0.05$, Fig. 4B). These results indicated that the free state of these four proteins significantly inhibited the phagocytosis of SEZ by macrophages.

Rabbit polyclonal antisera of SlaB and EndoS were successfully prepared (Fig. S1). In the anti-phagocytosis assay of wild-type ATCC35246, the strain was incubated with an equal volume of PBS, negative serum, anti-SlaB polyclonal antiserum, or anti-EndoS polyclonal antiserum, before incubation with macrophages. The results showed that wild-type ATCC35246 pre-incubated with anti-SlaB polyclonal antiserum or anti-EndoS polyclonal antiserum had significantly reduced anti-phagocytic ability compared to wild-type ATCC35246 pre-incubated with PBS or negative serum ($p < 0.05$, Fig. 4C).

The viability of M*htpZ*, M*spuZ*, M*slaB*, and M*endoS* in whole blood was significantly lower than that of the wild-type ATCC35246 ($p < 0.05$, Fig. 5). The viability of CM*htpZ*, CM*spuZ*, CM*slaB*, and CM*endoS* in whole blood was significantly higher than that of the corresponding transposon mutants ($p < 0.05$, Fig. 5). Except for CM*spuZ*, the viability of other complementary strains in whole blood was not significantly different from that of wild-type ATCC35246 ($p > 0.05$, Fig. 5). These results indicated that HtpZ, SlaB, SpuZ and EndoS were important for the survival of SEZ in whole blood.

4. Discussion

The identification of microorganisms by phagocytes during phagocytosis can be a direct process: for instance, pattern recognition receptors may identify large molecules on bacterial surfaces. Microorganism identification can also be indirect: for instance, when

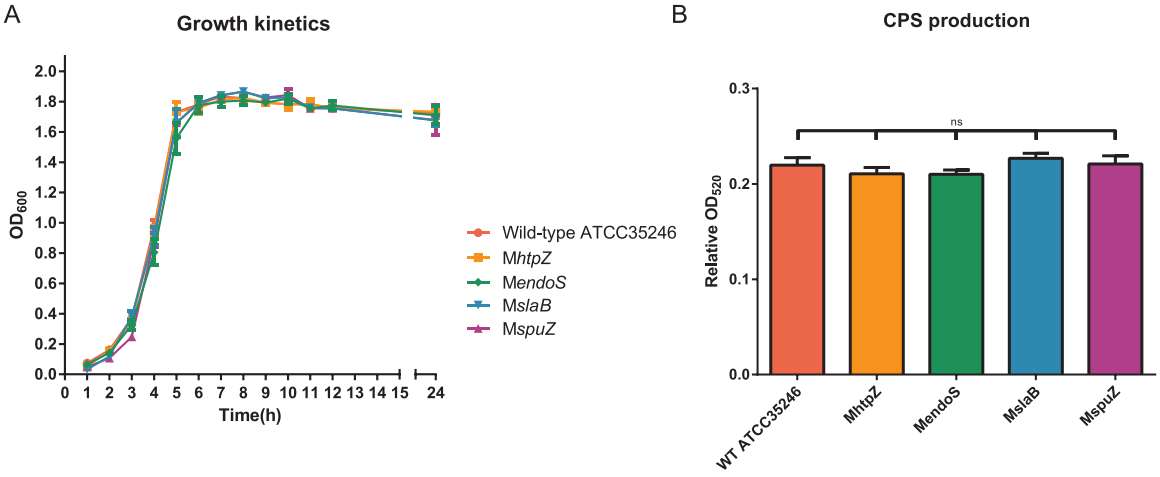

Fig. 3. Strain growth kinetics and capsule production. (A) Wild-type ATCC35246 and its transposon mutants were cultured aerobically in TH broth at 37 °C. The OD_{600} of the culture was measured every hour. Data represent means and standard deviations of the results of three independent experiments. (B) Quantification of capsule production by wild-type ATCC35246 and its transposon mutants. The statistical significance of differences between each pair of strains was determined using Student's *t* test (ns, not significant).

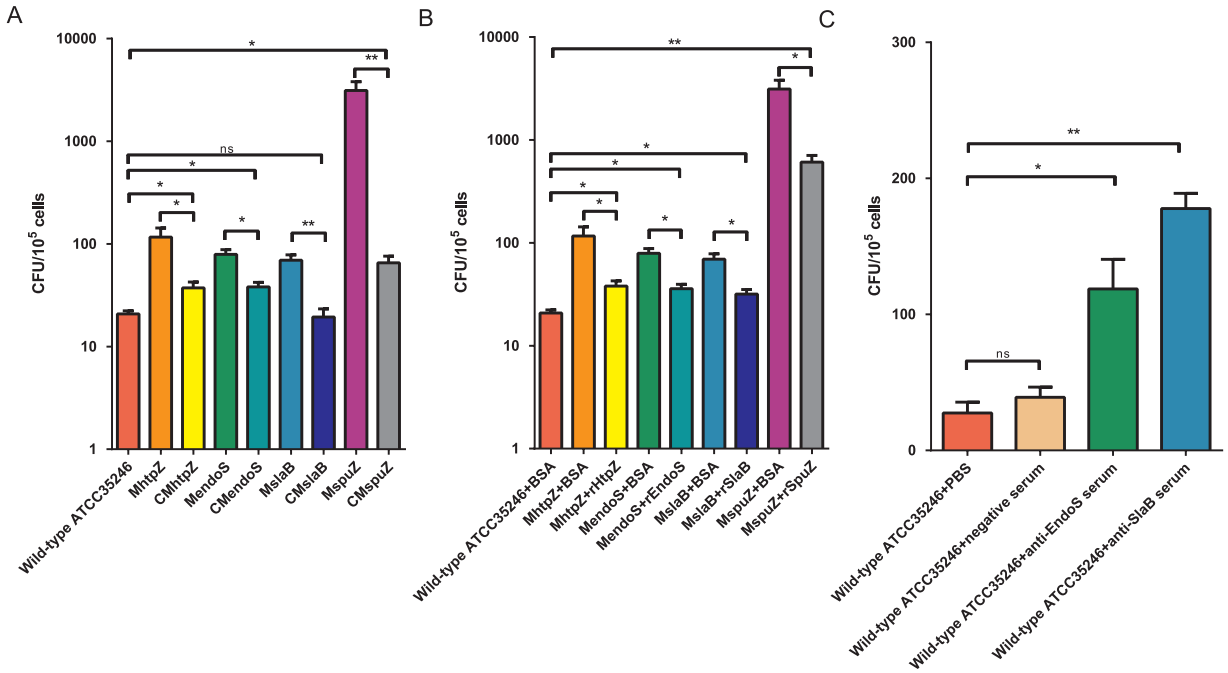

Fig. 4. Anti-phagocytosis of wild-type ATCC35246 and its transposon mutants. (A) CFUs of wild-type ATCC35246, its transposon mutants, and corresponding complementation strains recovered after phagocytosis per 1×10^5 Raw264.7 mammalian cells. (B) CFUs of wild-type ATCC35246, its transposon mutants, and strains incubated with BSA or the appropriate recombinant proteins recovered after phagocytosis per 1×10^5 Raw264.7 cells. (C) CFUs of wild-type ATCC35246 or wild-type ATCC35246 pre-incubated with negative serum or anti-protein serums recovered after phagocytosis per 1×10^5 Raw264.7 cells. Data represent means and standard errors of three independent experiments. The statistical significance of difference between each pair of strains was determined using Student's *t* test (*, $p < 0.05$; **, $p < 0.01$; ns, no significance).

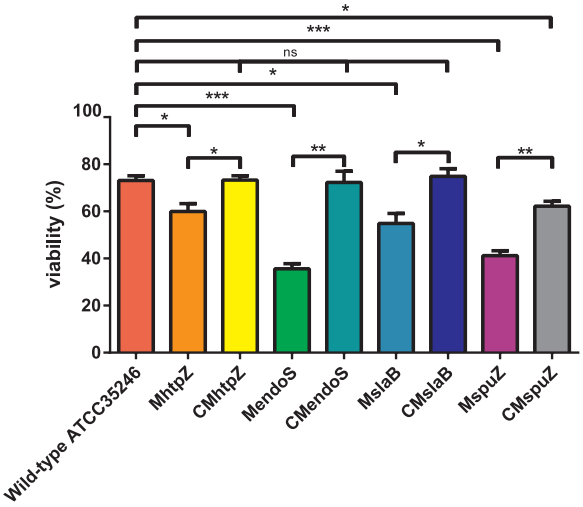

Fig. 5. Survival assays in whole blood. Wild-type ATCC35246, its transposon mutants, and corresponding complementation strains were grown to mid-log phage, harvested, and incubated with whole blood. Data represent means and standard errors of three independent experiments. The statistical significance of differences between each pair of strains was determined using Student's *t* test (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ns, no significance).

host opsonins, such as IgG or complement proteins, are deposited on bacterial surfaces and are then recognized by antibodies or complement receptors on the surface of the phagocytic cells (Flannagan et al., 2009). Macrophages activated by recognition undergo a series of signal transductions that are often dominated by Rho GTPase. Activated macrophages perform actin polymerization, pseudopod formation, cell membrane invagination, and microbial internalization at sites in contact with microorganisms (Caron and Hall, 1998). Thus, the interference with, or even blockage of, any of these processes by pathogenic microorganisms may increase pathogen resistance to phagocytosis. It has been suggested that SEZ may inhibit opsonin-mediated phagocytosis via multiple virulence factors, which are described in the introduction section. However, the SEZ virulence factors that interfere with the direct recognition of bacteria by macrophages or with the internalization process after recognition remain unknown. Therefore, we screened the SEZ virulence-related factors that might affect pathogen recognition or internalization, in the absence of opsonin.

By screening the random transposon mutant library, we found that various SEZ components were involved in the anti-phagocytic process. Among the proteins expressed by the mutant genes that were monocistronic or were the last gene located in a polycistron arranged in the direction of the transcription, there were many known virulence factors, including capsule (M4), SzM (M127), pilus protein YhgE (M259), and lipoprotein (M11); GtrA-like protein associated with bacterial surface polysaccharide synthesis (M401) (Kolly et al., 2015); proteins involved in gene transposition (M464 and M640); ABC transporters (M383, and M567); a sucrose operon repressor (M471); multiple types of enzymes, including those related to protein or amino acid metabolism (M45, M139, and M446), carbohydrate metabolism (M172, M231, and M554), lipid metabolism (M283), and nucleotide metabolism (M420, and M960); and conserved hypothetical proteins (M100, M206, M717, M810, M904). PurD (M420) was also identified for *S.pyogenes* fitness in human blood (Le Breton et al., 2013). Besides, the nucleotide biosynthesis has been shown to be critical for growth in blood for a variety of bacteria including *E.coli*, *Salmonella enterica*, and *Bacillus anthracis* (Samant et al., 2008). Some of proteins involved in the nucleotide biosynthesis such as PurD (M420) and GuaA (M960) were for the first time identified for SEZ fitness in phagocytosis resistance here.

Although only one gene was mutated by insertion in the remaining polycistronic mutants, the insertion of the TnYLB-1 transposon (contained a Rho-independent termination sequence) in upstream gene may reduce downstream gene transcription, or even silence downstream genes. Of these, M687 may have transcriptional disorders in six genes

(SeseC_00119 to SeseC_00125) encoding the late competence proteins. Late competent proteins are associated with multiple functions, including bacterial density regulation, microbial superiority support, horizontal gene transfer, and overall metabolic level. But their involvement in anti-phagocytic ability has not been shown. (Claverys et al., 2006). In M316, genes affected by the insertion mutation included two pili protein-encoding genes, two sortase C family protein-encoding genes. Gram-positive bacterial sortase C has been reported immobilizes anti-phagocytic-associated pilins on the bacterial surface (Hendrickx et al., 2011).

We focused on four transposon mutants: M*htpZ* (M11), M*spuZ* (M45), M*slaB* (M283), and M*endoS* (M554). The measurement of growth kinetics and capsular production showed that the decrease in anti-phagocytic ability was independent of the metabolic level and of the capsule. SEZ lipoprotein (SeseC_00825) is a histidine triad protein (HTP) family protein, and was this named HtpZ. HTP family proteins, as outer membrane proteins, are involved in the pathogenesis of multiple pathogenic streptococci, including *S. pneumoniae*, *S. pyogenes*, *S. agalactiae*, and *S. suis*, with the functions of zinc ion transport, evasion of complement deposition, adhesins, and protective antigens (Shao et al., 2013). In this study, the loss of the HtpZ was found significant decreased the anti-phagocytic abilities of SEZ in the absence of opsonin.

SpuZ, SlaB, and EndoS are three secreted enzymes with peptidase, phospholipase, and endoglycosidase activity, respectively (Sitkiewicz et al., 2006; Navais et al., 2014; Collin et al., 2002). SpuZ is a SEZ peptidase U32 family protein. It has been found that the U32 peptidase family proteins are associated with collagenase activity in a variety of Gram-negative bacteria such as *Proteus mirabilis* and *Salmonella enterica*, which is therefore considered to be involved in the bacterial infection (Navais et al., 2014). SEZ and SEE genomes both encode two homologous phospholipase A_2, SlaA and SlaB. *slaA* is presented only in 31% of tested SEZ isolates and all the tested SEE whilst *slaB* is present in all the tested SEZ and SEE isolates (Holden et al., 2009). ATCC35246 strain only presents *slaB*. Both SlaA and SlaB are immunogenic during natural SEE infection on horse, but they are not essential for the development of strangles in Welsh mountain ponies (Lopez-Alvarez et al., 2017). SlaA and SlaB share 98% and 70% amino acid sequence identity with SlaA of *S. pyogenes* (Holden et al., 2009). Studies have shown that SlaA can mediate the adhesion of *S. pyogenes* to cells, enhance *S. pyogenes* pathogenicity in mice, and increase bacterial cytotoxicity after invasion to cells (Sitkiewicz et al., 2006). *S. pyogenes* EndoS weakens the binding of the macrophage and neutrophil Fc receptors to IgG, and also reduces the deposition and activation of complement on the bacterial surface via the deglycosylation of IgG, which enhances the resistance of the bacteria to opsonophagocytosis (Collin et al., 2002). The enhancement of the anti-phagocytic activity of these three secreted proteins in the absence of opsonin is reported for the first time in this study.

The diseased pigs infected with SEZ often showed respiratory disturbances and painful swelling of the joint. Many of them died within a few days. The postmortem examination of the pigs revealed signs of septicemia, which showed bleeding in multiple organs, as well as bronchopneumonia, polyarthritis, meningitis, pleuritis, epicarditis, and endocarditis (Barnham et al., 1987; Soedarmanto et al., 1996). ICR mouse is highly susceptible to infection by wild-type ATCC35246 as the LD_{50} is about 300 CFUs. The LD_{50} of the avirulent SEZ strain ST_{171} in ICR mouse is about 5×10^4 fold increase when compared with that of wild-type ATCC35246 (Table 5). ICR mouse infected usually showed messy coat, difficulty in breathing, listlessness, and loss of appetite. Acutely dying ICR mice infected with wild-type SEZ often do not show clinical symptoms. The postmortem examination of the ICR mouse revealed signs of septicemia, such as bleeding in lungs, liver, heart, and spleen (unpublished data). The high lethality in mice of virulent strain, low lethality in mice of vaccine strain, and similar clinical symptoms and pathological changes in mice make artificial infection model of mice can simulate the natural disease in pigs infected with virulent SEZ.

In this study, we found that the decline in the anti-phagocytic abilities of SEZ often reduced the survival ability of SEZ in whole blood, and reduced mortality in mice post SEZ infection. In addition, our current and previous work demonstrated that commercial vaccine ST_{171} strain significantly reduced its anti-phagocytic ability and pathogenicity in mice compared to wild-type strains, respectively (Peng et al., 2017). These suggested that the anti-phagocytic abilities of SEZ are critical to its virulence in mice and even in pigs. The discovery of this variety of bacterial anti-phagocytic factors in this study enriches our understanding of the way bacteria respond to an innate immune attack by the host, providing rich material for further research on bacterial pathogenesis and related vaccine development for novel potential targets.

Conflict of interest statement

The authors have not declared any conflict of interest.

Acknowledgements

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2019.04.023.

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