

# *Streptococcus suis* Serotype 2 Capsule In Vivo

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Many *Streptococcus suis* isolates from porcine endocarditis in slaughterhouses have lost their capsule and are considered avirulent. However, we retrieved capsule- and virulence-recovered *S. suis* after in vivo passages of a non-encapsulated strain in mice, suggesting that nonencapsulated *S. suis* are still potentially hazardous for persons in the swine industry.

*Streptococcus suis* is a gram-positive bacterium that infects pigs and causes severe economic losses to the swine industry. Moreover, it causes severe disease in persons in close contact with diseased pigs or their products (1). In Japan, *S. suis* has been frequently isolated from pigs with endocarditis in slaughterhouses; most of the isolates were expected to be sequence types (STs) that are potentially hazardous to humans (2). Many isolates from porcine endocarditis lost their capsule, and all the nonencapsulated isolates analyzed had mutations in the capsular polysaccharide synthesis (*cps*) genes (3,4). The capsule of *S. suis* is a major virulence factor (1). Although loss of the capsule gives *S. suis* some benefit in causing endocarditis by enhancing the ability of bacterial cells to adhere to porcine and human platelets, a major virulence determinant for infective endocarditis (3), nonencapsulated *S. suis* are generally considered avirulent (5). However, whether nonencapsulated *S. suis* lurking in porcine endocarditis poses a threat to persons working in the swine industry is unknown. To investigate whether nonencapsulated *S. suis* can restore the ability to express the capsule and become virulent again, we repeated in vitro or in vivo passages of nonencapsulated *S. suis* and attempted to retrieve capsule-recovered strains.

## The Study

For the in vitro passages, we used 29 *S. suis* strains isolated from pigs with endocarditis. These isolates had the *cps* gene cluster of serotype 2 but had lost their capsule

because of mutations in the *cps* genes (Table). We subcultured them twice in liquid media and separated the cells according to the buoyant density by Percoll density gradient centrifugation (online Technical Appendix, <http://wwwnc.cdc.gov/EID/article/22/10/15-1640-Techapp1.pdf>). Because encapsulated cells show lower density than nonencapsulated cells (6,7), we investigated capsular expression of *S. suis* cells with low density by coagglutination tests using serotype 2 antiserum (online Technical Appendix). The retrieved *S. suis* was also used for the next subcultures. We repeated 4 cycles of this experiment (in total 8 subcultures) but obtained no encapsulated *S. suis* from any of the strains tested.

Although these results suggested that mutations in *cps* genes are not repaired easily, the conditions faced by *S. suis* in vivo could influence capsular expression. To investigate this possibility, we selected strain NL119 as a representative. NL119 is an ST1 strain, one of the types hazardous to humans, but one that has lost the capsule because of a point mutation that occurred at nt 490 (T490C, Cys164Arg) of a glycosyltransferase gene (*cps2F*) (Table; Figure 1, panel A) (4). We inoculated groups of 5 mice with  $5 \times 10^8$  CFU of NL119 (online Technical Appendix). Bacteria persistent in mice were retrieved 36 h after infection from the blood, in which capsular expression works favorably for survival. We investigated capsular expression of the retrieved NL119 by coagglutination tests and used the colony giving the strongest reaction within 30 s for the subsequent in vivo passage.

As expected, the coagglutination test of the parental strain NL119 showed a negative result. Similarly, NL119 after the first and second passages (NL119 P1 and P2, respectively) reacted weakly, comparable to those of the parental strain, suggesting poor encapsulation. Meanwhile, NL119 after the third and fourth passages (NL119 P3 and P4, respectively) reacted strongly, suggesting recovery of the capsule. To confirm this finding, we further analyzed formalin-killed bacteria by dot-ELISA using monoclonal antibody Z3, which reacts with the sialic acid moiety of the serotype 2 capsule (8), and an anti-*S. suis* serotype 2 serum adsorbed with parental strain NL119 to select the capsule-specific antibodies (online Technical Appendix). In accordance with the coagglutination test, NL119 P1 and P2 gave weak reactions similar to those of NL119, whereas strong signals were detected in NL119 P3 and P4 with both the monoclonal antibody and serum (Figure 1, panels B, C). Because NL119 P1–P4 were also ST1 as determined by multilocus sequence

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**Table.** Nonencapsulated *Streptococcus suis* strains isolated from pigs with endocarditis and used for in vitro passages to investigate possible capsule recovery

Strain	Affected gene(s)	Types of mutations	Affected nucleotide(s) (affected amino acid)	Reference
NL100	<i>cps2F</i>	Nonsense	T696G (Tyr232TERM)	(4)
NL119	<i>cps2F</i>	Missense	T490C (Cys164Arg)	(4)
NL122	<i>cps2F</i>	Missense	G52A (Gly18Ser)	(4)
NL126	<i>cps2F</i>	Frameshift by insertion	TCCG	(4)
NL132	<i>cps2E</i>	Missense	G1199A (Arg400Lys)	(4)
	<i>cps2H</i>	Frameshift by deletion	TA	(4)
NL143	<i>cps2F</i>	Missense	G493T (Asp165Tyr)	(4)
	<i>cps2K</i>	Insertion	AATCATTGG	(4)
	<i>cps2R</i>	Missense	G496A (Gly166Arg)	(4)
NL146	<i>cps2F</i>	Nonsense	T482A (Leu162TERM)	(4)
NL171	<i>cps2E</i>	Insertion	IS element: 1,619 bp	(3)
NL174	<i>cps2H</i>	Frameshift by deletion	A	(4)
NL175	<i>cps2H</i>	Frameshift by deletion	A	(4)
NL184	<i>cps2E</i>	Insertion	IS element: 1,115 bp	(3)
NL194	<i>cps2E</i>	Insertion	IS element: 1,416 bp	(3)
NL208	<i>cps2E</i>	Frameshift by deletion	TAAG	(4)
NL219	<i>cps2E</i>	Frameshift by deletion	TAAG	(4)
NL225	<i>cps2F</i>	Frameshift by insertion	CCAAA	(4)
NL230	<i>cps2F</i>	Frameshift by insertion	A	(4)
NL240	<i>cps2E</i>	Nonsense	C1189T (Gln397TERM)	(4)
NL245	<i>cps2E</i>	Frameshift by insertion	T	(4)
NL249	<i>cps2E</i>	Frameshift by insertion	AGCA	(4)
NL255	<i>cps2E</i>	Insertion	IS element: 1,619 bp	(3)
NL257	<i>cps2E</i>	Frameshift by insertion	ATCT	(4)
NL266	<i>cps2E</i>	Frameshift by deletion	A	(4)
NL278	<i>cps2F</i>	Missense	T259C (Ser87Pro)	(4)
NL295	<i>cps2F</i>	Missense	T492G (Cys164Trp)	(4)
NL303	<i>cps2F</i>	Deletion	81 bp	(4)
NL322	<i>cps2B</i>	Missense	G469A (Asp157Asn)	(4)
	<i>cps2G</i>	Deletion	50 bp	(4)
NL328	<i>cps2F</i>	Frameshift by deletion	AG	(4)
NL342	<i>cps2E</i>	Frameshift by deletion	TAAG	(4)
NL345	<i>cps2H</i>	Deletion	23 bp	(4)
	<i>cps2N</i>	Missense	C706T (Pro236Ser)	(4)

typing, these results suggested that NL119 had recovered the capsule during passages in animals.

To find mutations that had contributed to the capsule recovery, we sequenced the *cps2F* gene of NL119 P1–P4. Although the cytosine residue at nt 490 was not changed in comparison with the parental strain, we found a further missense mutation at nt 491 (G491C, Arg164Pro) of the *cps2F* gene in NL119 P3 and P4 (Figure 1, panel A). To investigate whether this mutation was involved in the capsule recovery, we cloned *cps2F* of NL119 P4 into a gene expression vector pMX1 (9) and introduced it into the parental strain NL119 (online Technical Appendix). A coagglutination test using serotype 2 antiserum showed positive reactions in all transformants tested, demonstrating that the further missense mutation restored the function of *cps2F*, resulting in capsule recovery of NL119 P3 and P4, although how the Cps2F function was recovered by the amino acid substitution is unknown. Isolation of the capsule-recovered strains in vivo could have been the consequence of selection of encapsulated cells, which were already present as a subpopulation in the original nonencapsulated NL119 population, by resisting host immunity including phagocytosis. However, because NL119 was a nonencapsulated strain

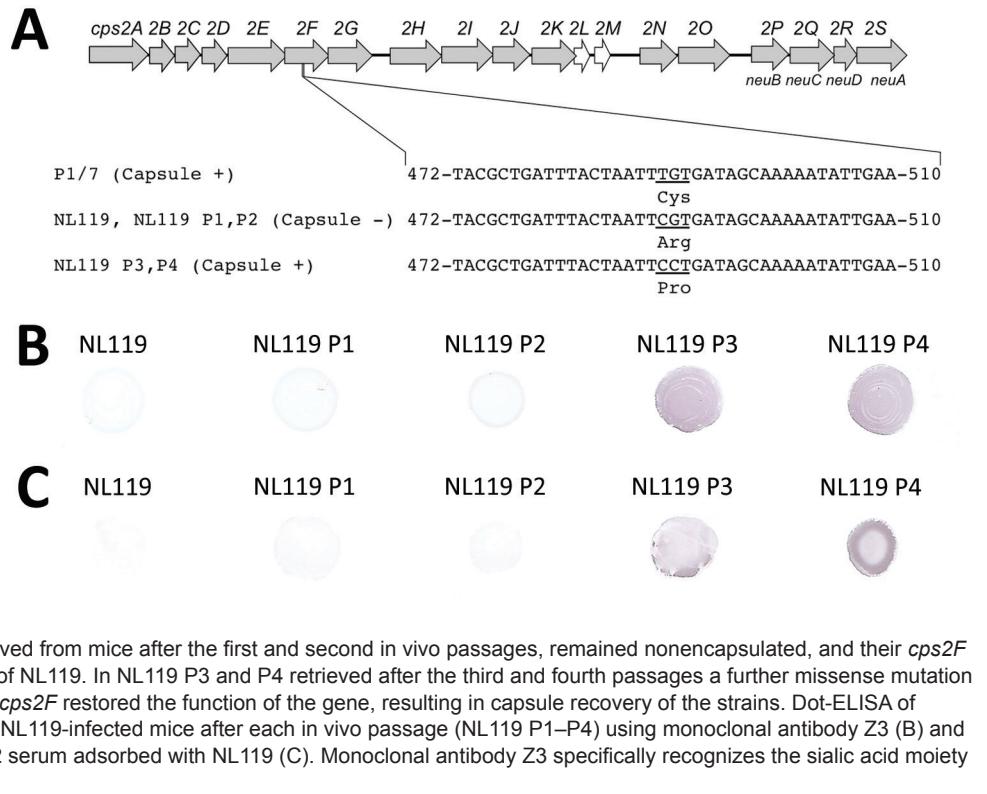
originally recovered from a single colony and well-isolated by repeated passages in vitro, and no encapsulated subpopulation was ever retrieved in vitro by the selection using Percoll density gradient centrifugation, the most plausible hypothesis would be that the capsule-recovered *S. suis* was generated in vivo.

To evaluate whether the capsule-recovered *S. suis* isolate also recovered its virulence, we infected mice with either NL119 or NL119 P4 (online Technical Appendix). Rates of death differed significantly ( $p < 0.05$ ): 50% death in the NL119 P4-infected mice 14 days after infection, compared with 0% for the nonencapsulated NL119 (Figure 2, panel A). Recovery of the capsule also significantly increased its survival in blood 24 h after infection ( $p < 0.05$ ). All but 1 surviving NL119 P4-infected mice had significant blood bacterial titers ( $\geq 5 \times 10^3$  CFU/mL; geometric mean  $10^4$  CFU/mL). In contrast, except for 1 mouse, all mice infected with NL119 had blood bacterial titers  $< 10^4$  CFU/mL (geometric mean  $10^2$  CFU/mL) (Figure 2, panel B).

## Conclusions

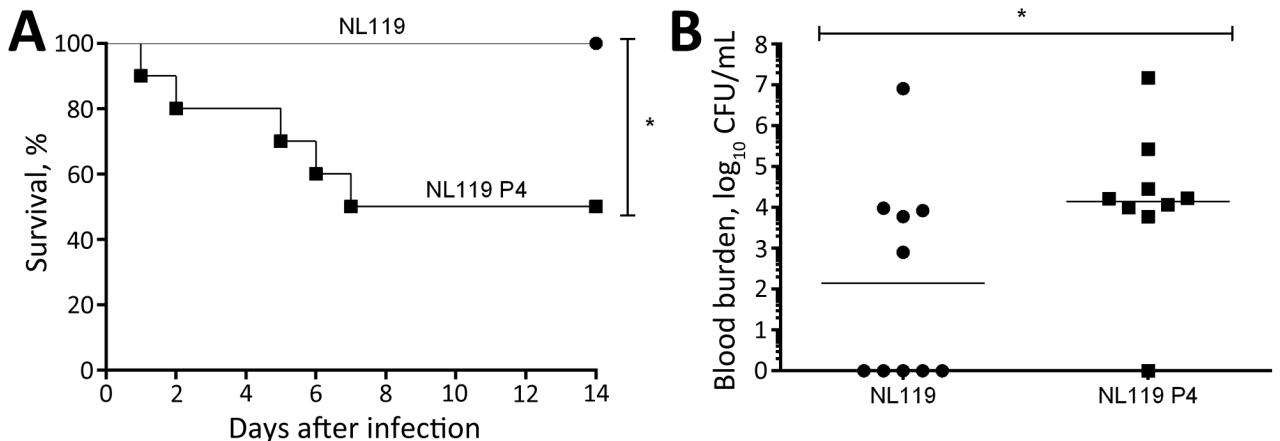
Although capsule loss might contribute to *S. suis* infection by enhancing bacterial adherence to host cells and

**Figure 1.** Capsule recovery of *Streptococcus suis* strain NL119 in vivo. A) The genetic organization of the *S. suis* serotype 2 capsular polysaccharide synthesis (*cps*) gene cluster and mutations observed in isolate NL119 and strains retrieved from NL119-infected mice after each in vivo passage (NL119 P1–P4; DDBJ/EMBL/GenBank accession nos. LC147077, LC147078, LC147079, LC147080, and LC077855, respectively). Gray arrows indicate genes putatively involved in capsule synthesis; open arrows indicate genes with unknown functions; numbers indicate nucleotide positions in *cps2F*. NL119 lost the ability to synthesize the capsule because of a missense mutation at nt 490 (T490C, Cys164Arg) of *cps2F* (4). B, C) NL119 P1 and P2 retrieved from mice after the first and second in vivo passages, remained nonencapsulated, and their *cps2F* sequences were identical to that of NL119. In NL119 P3 and P4 retrieved after the third and fourth passages a further missense mutation at nt 491 (G491C, Arg164Pro) of *cps2F* restored the function of the gene, resulting in capsule recovery of the strains. Dot-ELISA of NL119 and strains retrieved from NL119-infected mice after each in vivo passage (NL119 P1–P4) using monoclonal antibody Z3 (B) and polyclonal anti-*S. suis* serotype 2 serum adsorbed with NL119 (C). Monoclonal antibody Z3 specifically recognizes the sialic acid moiety of the *S. suis* serotype 2 capsule.



biofilm formation (3,10–12), capsule loss makes *S. suis* cells susceptible to phagocytosis; therefore, the virulence of nonencapsulated mutants was attenuated when evaluated in animal models (5). In accordance with previous studies, nonencapsulated NL119 was avirulent. However, NL119 P4, which recovered its capsule in vivo, also recovered virulence. Because various mutations in *cps* genes, including large deletions and insertions,

cause capsule loss in *S. suis* (3,4), not all mutations will be repaired like NL119. However, our results demonstrated the presence of a nonencapsulated mutant, which can recover the capsule and virulence in vivo. Hence, nonencapsulated *S. suis* strains can cause severe diseases to the next hosts by recovering the capsule, which indicates that some nonencapsulated *S. suis* lurking in pigs with endocarditis are still potentially hazardous to



**Figure 2.** Virulence of nonencapsulated *Streptococcus suis* strain NL119 and capsule-recovered NL119 P4 in mice. A) Survival of C57BL/6 mice (n = 10 mice per strain; until 14 days after infection) inoculated intraperitoneally with  $5 \times 10^7$  CFU of either NL119 or NL119 P4. B) Blood bacterial burden at 24 h after infection. Data of individual mice are presented as log<sub>10</sub> CFU/mL with the geometric mean. Asterisks indicate a significant difference between NL119 and NL119 P4 (p<0.05).

persons handling such pigs and their products. Further investigations using a variety of naturally occurring and laboratory-derived mutants are needed for a comprehensive understanding of the biological significance and mechanisms of this phenomenon.

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## EID Podcast: *Shigella Sonnei* and Shiga Toxin

Shiga toxins (Stx) are primarily associated with Shiga toxin-producing *Escherichia coli* and *Shigella dysenteriae* serotype 1. Stx production by other shigellae is uncommon, but in 2014, Stx1-producing *S. sonnei* infections were detected in California. During June 2014–April 2015, 56 cases of Stx1-producing *S. sonnei* were identified, in 2 clusters. Continued surveillance of Stx1-producing *S. sonnei* in California is necessary to characterize its features and plan for reduction of its spread in the United States.



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# *Streptococcus suis* Serotype 2 Capsule In Vivo

## Technical Appendix

### Materials and Methods

#### **In vitro Passages of Nonencapsulated *Streptococcus suis* Strains in Culture Media and Selection of *S. suis* Cells with Low Buoyant Density**

Nonencapsulated *S. suis* strains isolated from heart valve vegetations of pigs with endocarditis (1,2) were cultured overnight in 5 mL of Todd-Hewitt broth (THB; Becton Dickinson, Sparks, MD, USA) or THB containing 2% glucose and 1.5% disodium phosphate (THGB) at 37°C (first subculture). A total of 150 µL culture was then inoculated into 5 mL of fresh THB or THGB and cultured at 37°C for 9–12 h (second subculture). The bacterial cells subcultured twice were collected by centrifugation at 3,500 rpm for 20 min and washed once with 1 mL of 0.15 mol/L NaCl. Because encapsulated bacterial cells show lower buoyant density than nonencapsulated cells (3,4), the washed *S. suis* cells were then separated according to the buoyant density by Percoll density gradient centrifugation as follows.

For preparation of a stock isotonic Percoll (SIP) solution, Percoll PLUS (GE Healthcare UK Ltd, Buckinghamshire, UK) was diluted with 1.5 mol/L NaCl in a ratio of 9:1, and the pH was adjusted to 7.0 with 1 mol/L HCl. The subcultured and washed *S. suis* cells were suspended with 100 µL of the undiluted Percoll and added to the bottom of a 15-mL centrifuge tube. Solutions of 20%, 40%, 60%, and 80% SIP were prepared by further dilution of the SIP with 0.15 mol/L NaCl. Two milliliters each of these solutions was gently layered onto the *S. suis* suspension in the tube to produce a step gradient with 80% SIP at the bottom and 20% SIP at the top. The tube was centrifuged at 2,600 × gravity for 20 min to separate bacterial cells according to the density.

After the centrifugation, most of the *S. suis* cells, which were considered to be still nonencapsulated, were concentrated at the interface between 60% and 80% SIP. To retrieve *S. suis* cells with a lower density (i.e., *S. suis* cells that might express the capsule), we removed 50 µL of

the solution from the interface between 20% and 40% SIP. The removed solution was spread on Todd-Hewitt agar (THA) plates and cultured overnight at 37°C in air plus 5% CO<sub>2</sub>. The capsular expression of colonies grown on the plates was evaluated by coagglutination tests using the serotype 2 antiserum (1). Five to 10 colonies on the THA plates were also used as an inoculum for the next 2 times subcultures, and capsular expression of the subcultured bacteria was evaluated again after Percoll density gradient centrifugation. We repeated this experiment 4 times for each strain (in total 8 subcultures for each strain).

### **Animal Ethics**

Animal experiments were performed at the Université de Montréal (St-Hyacinthe, QC, Canada). All the experiments were conducted in strict accordance with the recommendations and approved by the Université de Montréal Animal Welfare Committee guidelines and policies.

### **In vivo Passages of Nonencapsulated *S. suis* in Mice**

For in vivo passages, a well-established C57BL/6 mouse model of infection (5) was used. Nonencapsulated *S. suis* NL119 was passaged a total of 4 times in mice. For preparation of inocula, the strain was grown overnight on Columbia Agar supplemented with 5% sheep blood (Oxoid, Nepean, ON, Canada) at 37°C with 5% CO<sub>2</sub>. Five milliliters of THB was inoculated with NL119 grown on the agar and incubated for 8 h at 37°C with 5% CO<sub>2</sub>. Working cultures were prepared by inoculating 30 mL of THB with 10 µL of a 10<sup>-3</sup> dilution of the 8 h cultures and incubating for 16 h at 37°C with 5% CO<sub>2</sub>. Bacteria were washed twice with pH 7.3 phosphate-buffered saline, suspended in THB, appropriately diluted, and plated on THA to accurately determine bacterial concentrations.

Six- to 10-week-old C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME, USA) were acclimatized to standard laboratory conditions with unlimited access to water and rodent chow. The mice were then inoculated with 5 × 10<sup>8</sup> CFU of NL119 by intraperitoneal injection, and bacteria were isolated from the blood of the surviving mice 36 h after infection by collecting 5 µL blood from the tail vein and plating the proper dilutions on THA. Five mice were used for each of the 4 passages (20 mice total). After each in vivo passage, 5 colonies from each surviving mouse were individually inoculated in 5 mL of THB and incubated for 16 h at 37°C with 5% CO<sub>2</sub>. After incubation, bacteria were collected by centrifugation for 10 min at 3,300 × gravity and suspended in phosphate-buffered saline with 0.5% formalin. The capsular expression of these isolates was

evaluated by coagglutination tests (6). The colony giving the strongest reaction within 30 sec was used for the subsequent in vivo passage. Capsular expression of strains selected by coagglutination after each passage was also confirmed by dot-ELISA as previously described, using both monoclonal antibody Z3 supernatant, which reacts with the sialic acid moiety of the *S. suis* serotype 2 capsule, and polyclonal anti-*S. suis* serotype 2 rabbit serum (7,8). To selectively obtain antibodies against the capsule, rabbit serum was adsorbed with an overnight culture of strain NL119 for 1 h at 37°C with agitation, for a total of 6 cycles (8).

### **Multilocus Sequence Typing**

Multilocus sequence typing (MLST) was performed by sequencing 7 housekeeping genes, as described previously (9). The sequence types of NL119 P1-P4 were determined by comparing the determined sequences with those in the *S. suis* MLST database (<http://ssuis.mlst.net>).

### **Sequencing of *cps2F***

Genomic DNA of *S. suis* NL119 and NL119 P1-P4 was extracted as described previously (10). The entire *cps2F* gene was amplified by KOD FX (TOYOBO Co., Ltd, Osaka, Japan) and Ex Taq DNA polymerase (Takara Bio Inc., Kusatsu, Japan) with primers *cps2-F6* and *cps2-R6* (1) and directly sequenced by a BigDye Terminator v3.1 cycle sequencing kit using 3130xl Genetic Analyzer (Applied Biosystems, Tokyo, Japan). SEQUENCHER Ver. 5.2 (Gene Codes Corp., Ann Arbor, MI, USA) was used for assembly and analysis of the sequences.

### **Construction of NL119 P4-Derived *cps2F* Expression Vector and Complementation Analysis**

To construct the NL119 P4-derived *cps2F* expression vector, the appropriate gene region was amplified from the genomic DNA of strain NL119 P4 by PCR with the primers CPS2F-F3 (5'-TTGAGGATCCAGGGAAGTAAGTAAGACTCC-3') and CPS2F-R (5'-TGCCCCATAGAATTCTGCTCCAGCATGGAG-3'), digested by *Bam*HI and *Eco*RI, and cloned into the respective sites of pMX1 (11). After being introduced into *Escherichia coli* strain MC1061 (12), the direction and sequences of the cloned genes were verified by PCR and sequencing. The resultant expression vector was then introduced into nonencapsulated *S. suis* NL119 by the method described previously (13), and the restoration of capsular expression was examined by coagglutination tests.

## Experimental Infection of Mice to Investigate the Virulence of NL119 and NL119 P4

To compare the virulence of nonencapsulated and capsule-recovered strains,  $5 \times 10^7$  CFU of NL119 or NL119 P4 was administered to 6-week-old C57BL/6 mice (10 mice/group) by intraperitoneal injection. Mice were monitored at least 3 times daily until 72 h after infection and then twice daily until 14 days after infection for clinical signs and death. Bacteremia of the surviving mice was evaluated 24 h after infection by using blood collected from the tail vein. An unpaired *t* test and log-rank (Mantel-Cox) test were performed to compare blood bacterial burden and survival, respectively.  $p < 0.05$  was considered as the threshold for statistical significance.

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