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Capsular polysaccharide switching in *Streptococcus suis* modulates host cell interactions and virulence

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The capsular polysaccharide (CPS) of *Streptococcus suis* defines various serotypes based on its composition and structure. Though serotype switching has been suggested to occur between *S. suis* strains, its impact on pathogenicity and virulence remains unknown. Herein, we experimentally generated *S. suis* serotype-switched mutants from a serotype 2 strain that express the serotype 3, 4, 7, 8, 9, or 14 CPS. The effects of serotype switching were then investigated with regards to classical properties conferred by presence of the serotype 2 CPS, including adhesion to/invasion of epithelial cells, resistance to phagocytosis by macrophages, killing by whole blood, dendritic cell-derived pro-inflammatory mediator production and virulence using mouse and porcine infection models. Results demonstrated that these properties on host cell interactions were differentially modulated depending on the switched serotypes, although some different mutations other than loci of CPS-related genes were found in each the serotype-switched mutant. Among the serotype-switched mutants, the mutant expressing the serotype 8 CPS was hyper-virulent, whereas mutants expressing the serotype 3 or 4 CPSs had reduced virulence. By contrast, switching to serotype 7, 9, or 14 CPSs had little to no effect. These findings suggest that serotype switching can drastically alter *S. suis* virulence and host cell interactions.

Streptococcus suis is an important porcine pathogen and zoonotic agent causing septicemia, meningitis and many other diseases^{1–4}. This bacterium has evolutionarily adapted to pigs, with nearly 100% of carriage rate in the upper respiratory tract^{4,5}. *S. suis* strains are serotyped based on structural differences in the capsular polysaccharide (CPS)^{2,4}. So far, twenty-nine serotypes and twenty-seven additional novel CPS synthesis (*cps*) loci (NCL) were reported^{6–9}. Among these serotypes, serotype 2 is responsible for the majority of human clinical cases and is the most frequently isolated from diseased pigs². Serotypes 1/2, 3, 4, 7, 8, 9, and 14 are also frequently isolated from diseased pigs, although their distributions differ depending on the geographic location². Multilocus sequence typing (MLST) for *S. suis* has demonstrated genetic diversity within this species, with more than 1,000 sequence types, and several clonal complexes (CCs) potentially associated with diseases in humans and pigs^{2,6}. Accumulated serotyping and MLST data indicate the presence of different CCs in the population of serotype 2 strains (CC1, CC20, CC25, CC28, and CC104), and different serotypes in the respective CCs (e.g., CC1 include strains of serotypes 1/2, 1, 2, 8, 9, and 14 strains) [pubMLST: <http://pubmlst.org/ssuis/>]. Taken together, this suggests that serotype switching may occur between *S. suis* serotype 2 and different serotype isolates.

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Strain	Sero-type ^a	MLST ^b	Description	Reference
P1/7 (SS2)	2	ST1 (CC1)	Serotype 2 reference strain isolated from a pig with meningitis; genome completely sequenced	24
ΔCPS2tocat	UT	ST1 (CC1)	Non-encapsulated P1/7 mutant, in which <i>cps2</i> genes (<i>cps2A-cps2S</i>) were replaced with the <i>cat</i> cassette; chloramphenicol resistant	This study
SS2to3	3	ST1 (CC1)	Serotype-switched P1/7 mutant, in which <i>cps2</i> genes (<i>cps2A-cps2S</i>) were replaced with <i>cps3</i> genes (<i>cps3A-cps3N</i>); serotype 3	This study
SS2to4	4	ST1 (CC1)	Serotype-switched P1/7 mutant, in which <i>cps2</i> genes (<i>cps2A-cps2S</i>) were replaced with <i>cps4</i> genes (<i>cps4A-cps4Q</i>); serotype 4	This study
SS2to7	7	ST1 (CC1)	Serotype-switched P1/7 mutant, in which <i>cps2</i> genes (<i>cps2A-cps2S</i>) were replaced with <i>cps7</i> genes (<i>cps7A-cps7R</i>); serotype 7	This study
SS2to8	8	ST1 (CC1)	Serotype-switched P1/7 mutant, in which <i>cps2</i> genes (<i>cps2A-cps2S</i>) were replaced with <i>cps8</i> genes (<i>cps8A-cps8P</i>); serotype 8	This study
SS2to9	9	ST1 (CC1)	Serotype-switched P1/7 mutant, in which <i>cps2</i> genes (<i>cps2A-cps2S</i>) were replaced with <i>cps9</i> genes (<i>cps9A-cps9N</i>); serotype 9	This study
SS2to14	14	ST1 (CC1)	Serotype-switched P1/7 mutant, in which <i>cps2</i> genes (<i>cps2A-cps2S</i>) were replaced with <i>cps14</i> genes (<i>cps14A-cps14V</i>); serotype 14	This study
ΔCPS2	UT	ST1 (CC1)	Non-encapsulated P1/7 mutant, in which <i>cps2</i> genes (<i>cps2A-cps2S</i>) were deleted	This study
MO691	3	ST108 (CC94)	Field isolate from a lung of a diseased pig; donor of serotype 3 genome DNA	25
6407	4	ST54 (CC53/54)	Serotype 4 reference strain from a diseased pig; donor of serotype 4 genome DNA	
MO690	7	ST29 (CC25)	Field isolate from the brain of a pig with meningitis; donor of serotype 7 genome DNA	25
MO941	8	ST87 (CC87)	Field isolate from a lung of a diseased pig; donor of serotype 8 genome DNA	25
1016/10	9	ST16 (CC16)	Field isolate from the brain of a diseased pig with meningitis; donor of serotype 9 genome DNA	26
DAN13730	14	ST6 (CC1)	Serotype 14 reference strain from a human; donor of serotype 14 genome DNA	
MNCM50	2	ST104 (CC104)	Clinical isolate from a patient with pulmonary edema, the source of the <i>afuC</i> gene	19

Table 1. *S. suis* strains used in this study. ^a UT, untypeable. ^b ST, sequence type; CC, clonal complex.

The *S. suis* CPS is produced by the repetition of a defined oligosaccharide unit formed by a unique arrangement of various sugars¹⁰. Indeed, unique CPS structures of serotypes 1, 2, 3, 7, 8, 9, 14, 18, and 1/2 have been previously determined^{11–16} (Supplementary Fig. S1). Furthermore, previous studies have shown that more than 10 genes related to *S. suis* CPS synthesis are clustered on a genomic locus^{7–10}. Alongside, the *cps* gene clusters of serotypes 1 and 14 and serotypes 2 and 1/2 are almost identical¹⁰, with their CPS structure differing by the substitution of only a galactose (Gal) for a *N*-acetylgalactosamine (GalNAc)¹³ due to a single nucleotide polymorphism in the glycosyltransferase *cpsK* gene¹⁷. Except for these four serotypes, gene repertoires in the *cps* gene clusters greatly differ between serotypes^{7–10}, indicating that up-take of genomic DNA of different serotypes and replacement of *cps* gene cluster by homologous recombination, using flanking sequences of the clusters, is usually required for serotype switching. In *S. suis*, some strains are naturally transformable, with the competent state induced by competence gene products^{18,19}. Although serotype switching in *S. suis* has not yet been demonstrated, these findings suggest that replacement of the *cps* gene clusters may occur in strains in the competent state through up-take of genomic DNA of the other serotype strains from the environment.

Importantly, the serotype 2 CPS has been shown to play critical roles in protection against phagocytosis by innate immune cells and masking of bacterial surface proteins involved in host cell activation²⁰. In addition, several studies have demonstrated non-virulence of isogenic non-encapsulated serotype 2 mutants in murine and porcine models of infection²⁰. However, very little information is available regarding the CPS of other *S. suis* serotypes and is restricted to two studies on serotypes 9 and 14^{20,21}. Furthermore, comparing the virulence of strains from different serotypes is impossible due to the high genotypic variation between strains. Accordingly, it remains unclear whether *S. suis* serotype switching (i.e., differences in CPS structure) can affect host cell interactions and strain virulence, even though serotype switching may occur among *S. suis* strains.

In the present study, serotype-switched *S. suis* mutants were experimentally generated to investigate the impacts of CPS type on the host cell interactions and virulence in vivo. The mutants were switched from serotype 2, which is the most important in this species, to serotypes 3, 4, 7, 8, 9, and 14, which are frequently isolated from diseased pigs and found in several CCs with serotype 2 human isolates (CC1, CC20, CC25, CC28, and CC104). Generated mutants have allowed us to study the modulation of the pathogenesis of *S. suis* caused by serotype switching. Preliminary information was discussed in a recent review²², although no data associated with the findings have been provided so far.

Results

Generated serotype-switched *S. suis* mutants contain few mutations other than the *cps* locus. Six different serotype-switched mutants (SS2to3, SS2to4, SS2to7, SS2to8, SS2to9, and SS2to14) and non-encapsulated mutant ΔCPS2, from which the *cps* locus was deleted, were generated from the serotype 2 strain P1/7 (hereafter SS2) (Table 1, generated as illustrated in Supplementary Figs. S2 and S3). Serotype-switched mutants were confirmed to belong to the correct serotype using classical serological techniques²³.

Serotype switching had little effect on bacterial growth in vitro (Supplementary Fig. S4). Well-encapsulation of the serotype-switched mutants was confirmed by surface hydrophobicity and transmission electron microscopy

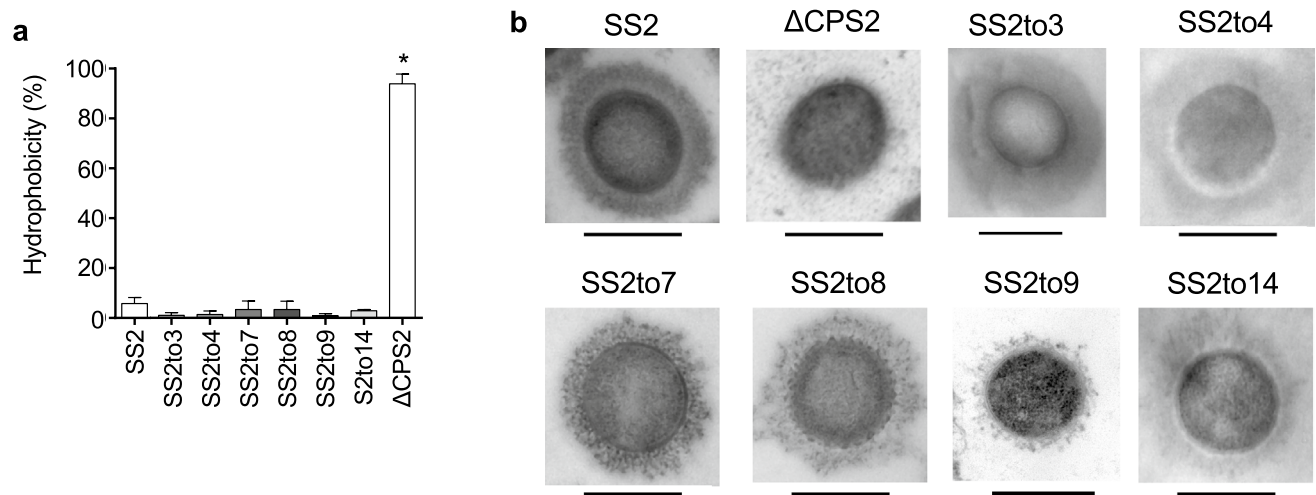


Figure 1. Effect of serotype switching on *S. suis* CPS expression. **(a)** Hydrophobicity of the different *S. suis* strains/mutants. Very low surface hydrophobicity is indicative of high encapsulation, which is demonstrated in the previous study²⁷. Data are expressed as mean \pm standard error of the mean (SEM) ($n = 3$). An asterisk denotes a significant difference with SS2 by Mann–Whitney rank sum test ($p < 0.05$). **(b)** Transmission electron micrographs showing CPS expression of the different *S. suis* strains/mutants. Scale bars = 0.5 μm .

(TEM) (Fig. 1A,B). Since the CPS repeating unit composition and structure for serotypes 2, 3, 7, 8, 9, and 14 have been previously determined^{12,14–16} (Supplementary Fig. S1), CPS of the mutants SS2to3, SS2to7, SS2to8, SS2to9, and SS2to14 were purified to be analyzed by spectroscopy. Purified CPS yields of the mutants were comparable to those previously reported^{12,14–16} (Supplementary Table S1). Nuclear magnetic resonance (NMR) analyses confirmed the serotype identity for the serotype-switched mutants, except for SS2to9 (Supplementary Fig. S5)^{12,14–16}. The CPS of SS2to9 slightly differed from that of serotype 9 strain 1,273,590 (used for CPS structure determination¹⁴) in that SS2to9 possessed a glucose instead of a galactose side chain (Supplementary Fig. S6a), suggesting that the donor strain and SS2to9 may be classified as a serotype 9 variant, which reacts with anti-serotype 9 serum (see Supplementary Notes for more detail). Taken together, these results confirm that the constructed serotype-switched mutants functionally possess and express the CPS of the donor serotype.

To investigate potential mutations in the genomes of the serotype-switched mutants occurred following the transformation of whole genomic DNA, draft genome sequences of the mutants were compared with those of SS2 and the donors. The mutants had mutations in several genes besides the *cps* genes, which differed between mutants (Fig. 2, Supplementary Fig. S7, and Supplementary Table S2; see Supplementary Notes for more detail). However, no genes other than *cps* genes were gained in the genomes of the different mutants. Comparison of the genomes of mutants with those of the corresponding donor strains revealed that the regions of the mutants that were different from the recipient were highly similar to the corresponding region of the donor (>99.7% of nucleotide identity) (Supplementary Table S3). Although it remains unclear whether these mutations might affect host–pathogen interactions and virulence, nonsense and frameshift mutations in genes, including reported virulence-associated genes³⁰, did not occur (Supplementary Table S2). In addition, no mutations were found in reported small RNAs²⁸. It should be noted that average nucleotide identity (ANI) between the recipient (strain SS2) and each the mutants was $\geq 99.9\%$ and the alignment coverage was $\geq 97.8\%$ (including the replaced *cps* locus), whereas ANI between the recipient and each the donor genomes was $< 98.0\%$ (with $< 92.8\%$ of the alignment coverage), except for the donor of SS2to14 (99.9% of ANI with 96.6% of the alignment coverage) (Supplementary Table S4). These data indicate that the mutants constructed in this study have almost identical genetic background to SS2 compared to the heterogenous genetic background of the different serotype strains, enabling more strict evaluation of the CPS effect hereafter.

Switching from serotype 2 of *S. suis* can modulate host cell interactions. The serotype 2 CPS has been described to mask surface adhesins involved in the initial interactions with host cells, including adhesion to and invasion of epithelial cells^{21,29}, to resist phagocytosis by macrophages and bactericidal killing by blood leukocytes to persist in the bloodstream and cause systemic dissemination²⁰, and to mask subcapsular immunostimulatory components to interfere pro-inflammatory mediator production by dendritic cells (DCs)^{30,31}.

First, using newborn pig trachea (NPTr) cells, the adhesion and invasion capacities were evaluated between SS2 and the mutants. While SS2, SS2to3, SS2to4, SS2to9, and SS2to14 similarly adhered to NPTr cells at 2 h, adhesion of SS2to7 and SS2to8 was significantly greater ($P < 0.05$), similar to that of ΔCPS2 used as a positive control (Fig. 3a). Unlike adhesion results, invasion of the different mutants was similar to that of SS2, with little invasion of NPTr cells overall, although ΔCPS2 showed high levels of invasion, as expected (Fig. 3b).

Next, macrophage phagocytosis resistance was evaluated using the J774A.1 murine macrophage cell line. As expected, SS2 and ΔCPS2 were poorly and highly internalized by macrophages, respectively (Fig. 3c). No differences were observed in the internalization between SS2 and the serotype-switched mutants after 1 h incubation (data not shown); however, switching to serotype 4, 7 or 8 significantly increased phagocytosis after 2 h

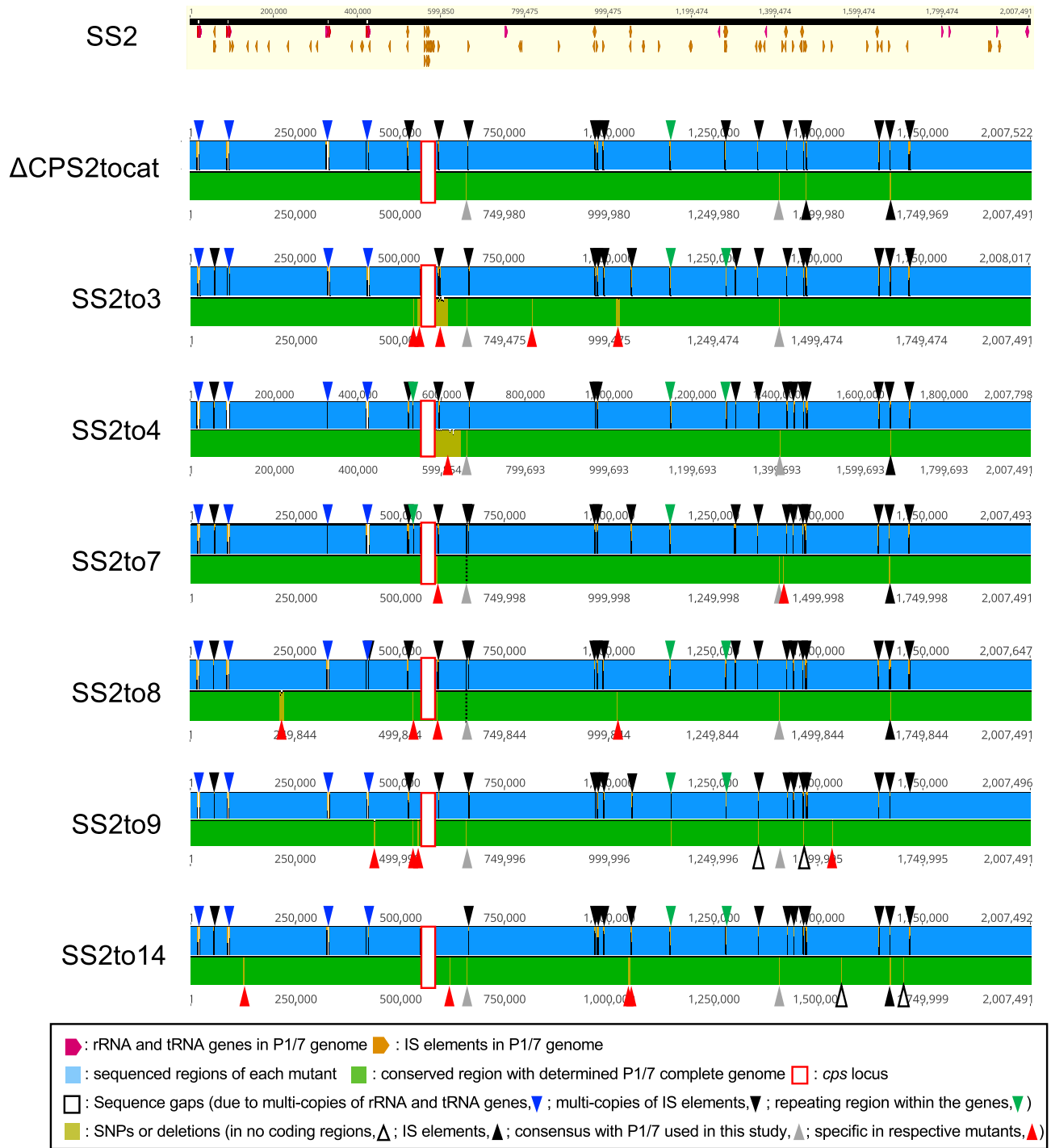


Figure 2. Mutations present in the generated *S. suis* serotype-switched mutants. Each of the schematic representations illustrates the analysis data using Geneious Prime mapping of the draft genome sequence of each mutant (upper part) on the publicly available completed genome sequence of serotype 2 (accession no. AM946016) and the sequence alignment between two genomes (lower part). All gaps between the contigs of each mutant were due to multi-copy genes, such as rRNA genes, tRNA genes and IS elements, or repeated regions within genes. Gaps of the repeated regions within genes were found in the genes corresponding to the SS2 locus tags SSU0496, SSU1127, SSU1171, and SSU1172. Detailed data on mutated genes can be found in Supplementary Table S2. Below the bottom panel are displayed the descriptions for each color of the different drawings.

incubation ($P < 0.05$) (Fig. 3c). However, it should be noted that this increase was of approximately one log-fold, which is, though significant, relatively minor compared to the non-encapsulated mutant (4 log-fold increase).

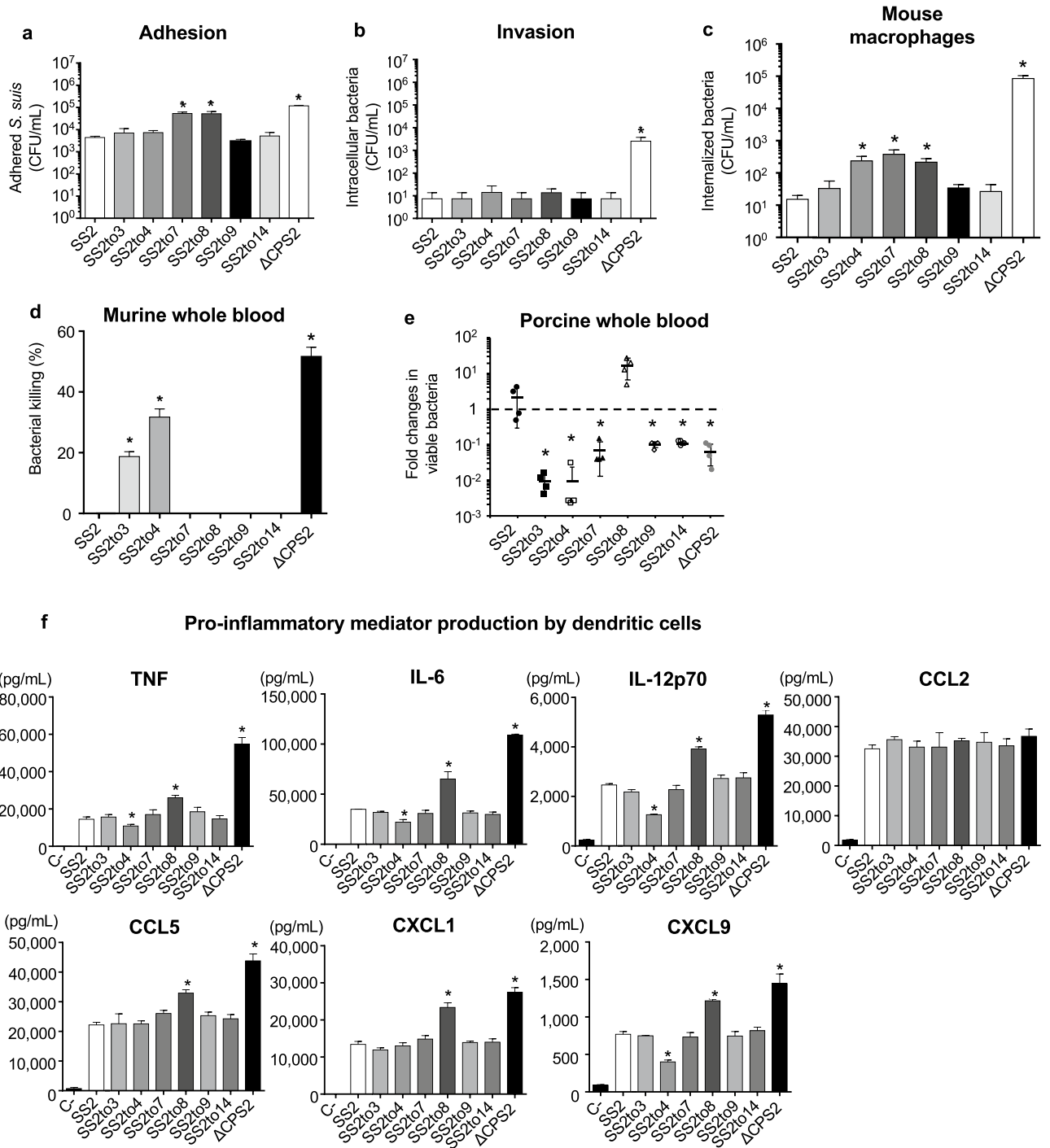


Figure 3. Impact of serotype switching on *S. suis* adhesion to and invasion of porcine tracheal epithelial cells, resistance to phagocytosis by macrophages, whole blood bacterial killing, and pro-inflammatory mediator production by dendritic cells. Adhesion (a) and invasion (b) of the different *S. suis* strains and mutants to NPT1 porcine tracheal epithelial cells after 2 h of incubation. (c) Internalization of the different *S. suis* strains and mutants by J774A.1 murine macrophages after 2 h of incubation. (d) Killing of the different *S. suis* strains and mutants by murine whole blood after 4 h of incubation. (e) Growth capacity of the different *S. suis* strains and mutants in porcine whole blood after 4 h of incubation. (f) Pro-inflammatory mediator production by DCs at 16 h following infection with the different *S. suis* strains and mutants as measured by ELISA. Production of tumor necrosis factor (TNF), interleukin (IL)-6, IL-12p70, C-C motif chemokine ligand (CCL) 5, and C-X-C motif chemokine ligand (CXCL) 1, and CXCL9. C-denotes cells in medium alone. All the data represent the mean ± SEM (n=4). An asterisk denotes a significant difference with SS2 by Mann–Whitney rank sum test (e) ($p < 0.05$).

The capacity to resist the bactericidal effect of leukocytes was then evaluated using murine and porcine whole blood. SS2 was completely resistant to killing by murine blood in contrast to Δ CPS2, which was efficiently killed (60% of killing) (Fig. 3d). While SS2to7, SS2to8, SS2to9, and SS2to14 were also resistant to killing by murine whole blood, SS2to3 and SS2to4 were significantly more killed, with 20% and 30% of killing, respectively ($P < 0.05$) (Fig. 3d). Using a porcine blood system, SS2 was not only able to persist, but also to some extent multiply, whereas Δ CPS2 was markedly cleared ($P < 0.05$) (Fig. 3e). Comparable to SS2, SS2to8 could significantly multiply, whereas all other mutants were cleared at different degrees (Fig. 3e). As with mouse blood, SS2to3 and SS2to4 showed the greatest impairment in their capacity to survive in porcine blood (Fig. 3e). It should be noted, however, that levels of cross-reactive antibodies against the different strains might affect the results observed with the swine blood and thus can be considered a confounding factor, although this fact also mimics the real situation in the field.

Lastly, the interactions with DCs were evaluated. Absence of CPS significantly increased production of all mediators tested ($P < 0.05$), with the exception of CCL2 (Fig. 3f), as previously reported^{21,29}. SS2to3, SS2to7, SS2to9, or SS2to14, along with SS2, did not modulate pro-inflammatory mediator production (Fig. 3f). However, stimulation with SS2to8 significantly increased production of TNF, IL-6, IL-12p70, CCL5, CXCL1, and CXCL9, compared to SS2 ($P < 0.05$) (Fig. 3f). By contrast, SS2to4 induced significantly lower levels of TNF, IL-6, IL-12p70, and CXCL9 than SS2 ($P < 0.05$), but CCL5 or CXCL1. CCL2 production was not modulated regardless of the CPS type (Fig. 3f).

Serotype switching can differentially modulate *S. suis* virulence in a mouse model of systemic infection.

The impact of switching from serotype 2 on *S. suis* virulence was evaluated using a well-established C57BL/6 mouse infection model for *S. suis* serotype 2 virulence studies³². Following intraperitoneal inoculation of SS2, 60% of mice died after developing clinical signs of systemic infection (Fig. 4a). By contrast, none of the Δ CPS2-inoculated mice died, presenting no or very mild clinical signs the first 24 h only (Fig. 4a). No significant differences in mortality were observed between SS2 and SS2to3, SS2to7, SS2to9 or SS2to14 (Fig. 4a). However, clinical signs of infection caused by SS2to3 were generally less severe than those by SS2. Unexpectedly, inoculation of SS2to8 significantly increased mouse mortality, with 100% of mice succumbing to septic shock within 24 h post-infection ($P < 0.05$) (Fig. 4a). By contrast, none of the SS2to4-infected mice died, presenting transient clinical signs within the first 48 h ($P < 0.05$) (Fig. 4a).

Blood bacterial burdens of infected mice were also determined to investigate the effect on persistent bacteraemia. Twenty-four hours post-infection, bacterial burdens of SS2-infected mice averaged 3×10^7 colony-forming unit (CFU)/mL, whereas those in mice infected with Δ CPS2 were not detectable ($< 1 \times 10^2$ CFU/mL) (Fig. 4b). Similar to mortality, no significant difference was observed between SS2 and SS2to3, SS2to7, SS2to9 or SS2to14 (Figs. 4b and S8). Meanwhile, blood bacterial burden of SS2to8-infected mice was significantly greater than that of SS2-infected mice ($P < 0.05$), averaging 2×10^9 CFU/mL (Fig. 4b). By contrast, blood bacterial burden was significantly reduced in SS2to4-infected mice compared to SS2 ($P < 0.05$), although blood burden remained detectable until at least 72 h post-infection, which differs from Δ CPS2-infected mice (Fig. 4b and Supplementary Fig. S8).

Furthermore, plasmatic levels of different pro-inflammatory mediators (12 h post-infection) were evaluated to investigate exacerbated systemic inflammation. The levels were elevated in SS2-infected mice, whereas they were undetectable in Δ CPS2-infected mice (Fig. 4c). Globally, no differences were observed in systemic inflammation between SS2-infected mice and those infected with SS2to7, SS2to9, or SS2to14 (Fig. 4c). However, a significant increase in the production of all the inflammatory mediators was observed in SS2to8-infected mice ($P < 0.05$), in accordance with the results on mortality observed above (Fig. 4a). Meanwhile, plasmatic levels of all mediators were significantly decreased in SS2to4-infected mice compared to SS2 ($P < 0.05$), although levels were detectable (Fig. 4c). Notably, infection with SS2to3 resulted in a significant reduction of most pro-inflammatory mediators compared to SS2, though reduction was not as great as with SS2to4 (Fig. 4c).

Serotype switching can differentially modulate *S. suis* virulence in piglets.

Impact of serotype switching on *S. suis* virulence was subsequently evaluated in the natural host of this bacterium by an experimental intranasal infection model, representing the natural route of exposure to *S. suis*. The mutants were divided into two experiments (experiment I: SS2, Δ CPS2, SS2to4, or SS2to7; experiment II: SS2, SS2to3, SS2to8, or SS2to14) (Table 2). Virulence of the SS2to9 was not evaluated for ethical reasons, since no differences were observed in host cell interactions assays in vitro nor in the mouse infection model. In experiment I, none of the Δ CPS2-infected pigs developed any clinical signs of infection, while all SS2-infected pigs showed clinical signs of systemic and/or central nervous system infection, including lame and shivering (Supplementary Table S5). In fact, three out of four SS2-infected pigs were euthanized at 3 or 4 days post-infection (dpi) due to severity of clinical signs (Table 2 and Supplementary Table S5). The inoculated strain was recovered from the blood and several organs, including the joints and brain, in all SS2-infected pigs (Table 3 and Supplementary Table S6). Recovery of SS2 from the joints and brain was also confirmed in the animals presenting lameness or shivering (Table 3 and Supplementary Table S6). Meanwhile, recovery of the inoculum was not observed from any of the investigated sites in the Δ CPS2-infected pigs, except for the tonsils (two pigs) and the liver (one pig) (Table 3 and Supplementary Table S6). All SS2to4- and three of SS2to7-infected pigs presented no clinical signs of infection (Table 2 and Supplementary Table S5), which were, except for the tonsils and a single organ, negative for bacterial recovery (Table 3 and Supplementary Table S6). However, one of the SS2to7-infected pigs developed shivering, and bacteria were only recovered from the brain and tonsils (Supplementary Table S6).

Unfortunately, none of the SS2-infected pigs developed clinical signs in experiment II, with recovery only from the tonsils and joints (Table 3 and Supplementary Table S6), although slight fever was observed 4 dpi

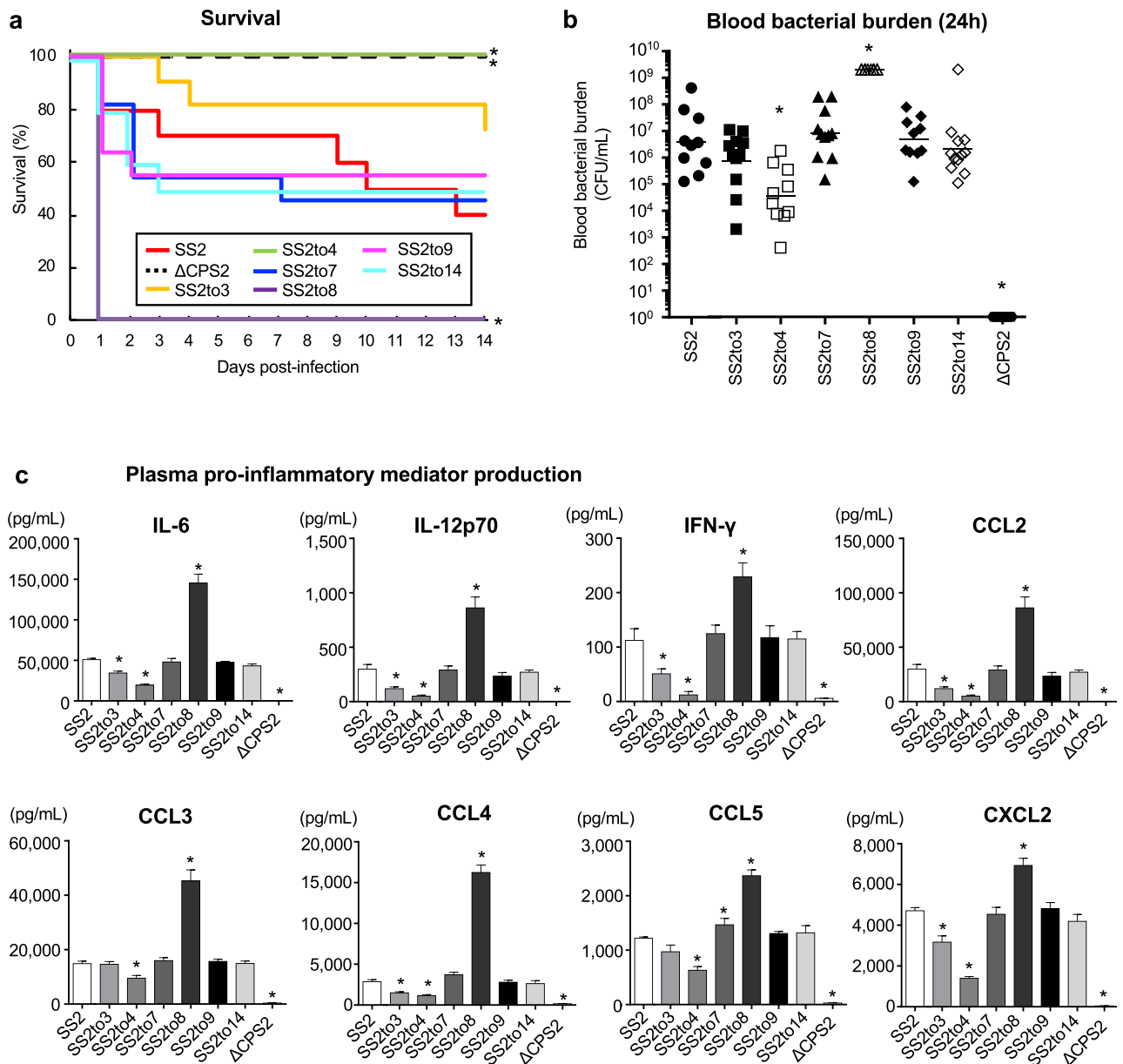


Figure 4. Impact of serotype switching on *S. suis* virulence and plasma pro-inflammatory mediator production in a mouse model of infection. **(a)** Survival of C57BL/6 mice following intraperitoneal inoculation of 1×10^7 CFU of the different *S. suis* strains and mutants. **(b)** Blood bacterial burden 24 h post-infection of C57BL/6 mice. A blood bacterial burden of 2×10^9 CFU/mL, corresponding to average burden upon euthanasia, was attributed to euthanized mice. **(c)** Plasma levels of IL-6, IL-12p70, IFN- γ , CCL2, CCL3, CCL4, CCL5, and CXCL2 in C57BL/6 mice at 12 h following intraperitoneal inoculation of 1×10^7 CFU of the different *S. suis* strains and mutants. Data represent survival curves **(a)** ($n = 10-12$), geometric mean **(b)** ($n = 10-12$) or mean \pm SEM **(c)** ($n = 8$). An asterisk denotes a significant difference with SS2 by Log-rank (Mantel-Cox) test **(c)** and Mann-Whitney rank sum test **(b and c)** ($p < 0.05$).

(Table 2 and Supplementary Table S5). These difference in results of SS2 between experiments may be due to the pigs being used originated from different suppliers. Although most SS2to3-, SS2to8-, or SS2to14-infected pigs showed no clinical signs, one of the SS2to8-infected pigs developed clinical symptoms, including inactivity and clear incoordination (Table 2 and Supplementary Table S5). Nevertheless, SS2to14 was recovered from the blood and organs of one of the infected pigs. Excluding this individual, however, bacterial recovery was mostly negative for SS2to3- or SS2to14-infected pigs. Meanwhile, bacteria were recovered from multiple organs in all the SS2to8-infected pigs, though recovery from blood was recorded in only the individual presenting clinical symptoms (Table 3 and Supplementary Table S6).

Exp. no.-Group no	Strain	Infection dose (CFU)	Mortality ^a	Morbidity ^b	Body temp > 40.5 °C	Description of clinical signs
I-1	SS2	2.0 × 10 ⁹	1/4	4/4	4/4	Lameness (3/4) Symptoms improved in one of the pigs Shivering with vomition (1/4)
I-2	ΔCPS2	2.9 × 10 ⁹	0/4	0/4	0/4	
I-3	SS2to4	2.8 × 10 ⁹	0/4	0/4	0/4	
I-4	SS2to7	3.1 × 10 ⁹	1/4	1/4	1/4	Shivering and clearly uncoordinated
II-1	SS2	1.4 × 10 ⁹	0/4	0/4	2/4	Slight fever at 4 dpi (2/4) Slight inactivity at 5 dpi (4/4) All animals subsequently recovered
II-2	SS2to3	2.8 × 10 ⁹	0/4	0/4	0/4	
II-4	SS2to8	1.2 × 10 ⁹	1/4	1/4	2/4	Inactive and lame
II-5	SS2to14	1.2 × 10 ⁹	0/5	0/5	0/5	

Table 2. *S. suis* swine infection outcomes and clinical diseases. ^aNumber of pigs to reach predefined clinical end point (see Supplementary Methods for more detail). ^bNumber of pigs having a score of > 1 on attitude or locomotion. Exp., Experiment; dpi, days post-infection.

Exp. no.-Group no	Strain	Morbidity	No. of pigs in which inoculum was recovered/total no. of pigs									
			Tonsil	Lung ^a	Kidney	Spleen	Liver	Brain ^b	Joint ^c	EC	Blood	Multiple organs ^d
I-1	SS2	4/4	4/4	1/4	1/4	4/4	2/4	2/4	3/4	1/4	4/4	4/4
I-2	ΔCPS2	0/4	2/4	0/4	0/4	0/4	1/4	0/4	0/4	0/4	0/4	0/4
I-3	SS2to4	0/4	4/4	0/4	0/4	0/4	1/4	0/4	0/4	0/4	0/4	0/4
I-4	SS2to7	1/4	4/4	0/4	0/4	0/4	0/4	1/4	0/4	0/4	0/4	0/4
II-1	SS2	0/4	4/4	0/4	0/4	0/4	0/4	0/4	2/4	0/4	0/4	0/4
II-2	SS2to3	0/4	4/4	0/4	1/4	0/4	2/4	0/4	0/4	0/4	0/4	1/4
II-3	SS2to8	1/4	4/4	1/4	2/4	3/4	3/4	1/4	1/4	4/4	1/4	4/4
II-4	SS2to14	0/5	5/5	1/5	1/5	2/5	2/5	1/5	1/5	1/5	1/5	2/5

Table 3. Recovery of inoculated strains from infected piglets. ^a Part of one cranial lobe was investigated. ^b Part of cerebrum was investigated. ^c Swab from a joint of the hind legs. In cases of lameness, a joint puncture of the corresponding limb was screened. ^d Recovery from two or more sites, except for tonsils. Exp., Experiment; EC, endocardium.

Discussion

In the present study, serotype-switched *S. suis* mutants were generated from serotype 2 to serotypes 3, 4, 7, 8, 9 or 14 via induction of competence state using XIP, which is the first report experimentally demonstrating full *cps* locus exchange in *S. suis*. CPS sugar composition and structure of the mutants were identical to those of the same serotype strains previously described in the literatures^{11–16}, except for the SS2to9 mutant, of which *cps* locus was identical to the donor serotype 9 strain used in this study but different from the strain previously used to determine the serotype 9 CPS structure¹⁴. In addition, our whole genome sequencing confirmed the deletion of the serotype 2 *cps* locus and the gain of the expected serotype *cps* locus of the respective donor strains, and revealed almost the same genetic background of each of the constructed mutant as SS2, enabling strict evaluation of the CPS effect alone; these findings suggest that replacement of the *cps* locus between the different serotypes alone was responsible for *S. suis* serotype switching. It should be noted, however, that it remains unclear whether several mutations, which were found in the genome other than *cps* locus of the respective mutants, affect the phenotype, including virulence and host cell interactions.

This study also provides the first evidence that serotype switch in *S. suis* can definitively modify the interactions with host cells and in vivo (Summarized in Table 4). CPS expression of *S. suis* serotypes 2, 9, and 14 plays critical roles on colonization and anti-phagocytic activity, important steps of the pathogenesis^{20,21,33}. In this study, only switching to serotype 7 or 8 changed the adhesion pattern of SS2 to porcine tracheal epithelial cells. The possibility that differential exposure of cell wall components, particularly adhesins, might be the explanation for the increase of *S. suis* adhesion is worth testing in the future. Differences in thickness of expressed CPS may also be responsible, although TEM results suggested similar thickness among mutants. It is also possible that a yet unknown host cell receptor might recognize certain motifs of specific *S. suis* CPSs. Moreover, results obtained in this study confirmed the previous speculation that the bacterial factors involved in *S. suis* adhesion and invasion probably differ and that the CPS itself is not involved in the latter²¹.

Strain	Serotype	In vitro						In vivo						
		Porcine NPTr cells		Murine macrophages	Murine DCs	Murine blood	Porcine blood	Mice			Pig (Exp. I)		Pig (Exp. II)	
		Adhesion	Invasion	Anti-phagocytosis	Pro-inflammatory mediator production	Resistance to killing	Mortality	Blood burden	Pro-inflammatory mediator production	Morbidity	Organ dissemination	Morbidity	Organ dissemination	
SS2to3	3	–	–	–	–	↓	↓	–	↓	↓	NT	NT	–	↑
SS2to4	4	–	–	– (↓) ^a	↓	↓	↓	↓	↓	↓	↓	↓	NT	NT
SS2to7	7	↑	–	– (↓) ^a	–	–	↓	–	–	–	↓	↓	NT	NT
SS2to8	8	↑	–	– (↓) ^a	↑	–	–	↑	↑	↑	NT	NT	↑	↑
SS2to9	9 (variant)	–	–	–	–	–	↓	–	–	–	NT	NT	NT	NT
SS2to14	14	–	–	–	–	–	↓	–	–	–	NT	NT	–	↑
ACPS2	Non-typable	↑	↑	↓	↑	↓	↓	↓	↓	↓	↓	↓	NT	NT

Table 4. Summary of the effects caused by serotype switching from serotype 2 on in vivo and in vitro virulence analyzed in this study. ^aAfter 2 h incubation, significantly more bacteria were internalized. NPTr, newborn pig trachea; DC, dendritic cell; –, no significant difference compared to SS2; ↑, significantly higher than SS2; ↓, significantly lower than SS2; NT, not tested.

Regarding anti-phagocytic activity, no significant (serotype 3, 9 or 14) or minor difference (serotype 4, 7, or 8) was observed by serotype switching, suggesting that the switch of CPS expressed at the *S. suis* surface may, at least partially, affect the anti-phagocytic properties conferred. A previous study³⁴ reported that serotype 4 and 7 wild-type strains were relatively more internalized by human monocyte-derived DCs than a serotype 2 strain, though the strains used have completely different genetic backgrounds. Here again, this study suggests the possibility that differences in phagocytosis is due to the exposure of differential cell wall component and/or activation of phagocytic receptors such as scavenger receptors and C-type lectins by specific CPS composition/structure. Indeed, C-type lectin receptors are involved in the uptake of *Streptococcus pneumoniae*^{35,36}, and their potential involvement in *S. suis* recognition remains to be evaluated.

Results obtained for the two parameters described above (adhesion to epithelial cells and phagocytosis by macrophages) provided the first evidence that the CPS composition/structure can definitively modify *S. suis* interactions with host cells. However, a single cell-type system does not accurately represent the complexity of the bacterial interplay with its host. By further evaluation of the effects on serotype-switching using ex vivo (blood) and in vivo infection models (mouse and pig), only mutants switched to serotype 4 or 8 showed a marked and consistent impact on several bacterial virulence traits. The CPS4 conferred to *S. suis* a non-virulent phenotype characterized by increased susceptibility to killing by mouse and pig blood, reduced bacteremia in mice, diminished cytokine production (in vitro and in vivo), and low bacterial recovery from internal organs in pigs. In marked contrast, the CPS8 conferred to *S. suis* an hyper-virulent phenotype characterized by high capacity to multiply in pig blood, high bacteremia (mice) and organ dissemination (pigs), and increased capacity to induce a cytokine storm (in vitro and in vivo in the mouse model).

It should be noted that switching to serotype 14 or 9 (variant) had no major effects on *S. suis* virulence or its interactions with the host either in vitro or in vivo in the mouse model. The results on CPS9 in this study makes a striking contrast with the previous study comparing serotypes 2, 14 and 9 with their corresponding non-encapsulated mutants²¹. Indeed, the CPS9 observation is somehow unexpected; it has been shown that the serotype 9 strain 1135776 adhered more to porcine tracheal epithelial NPTr cells, was more internalized by macrophages, and induced much lower in vitro pro-inflammatory mediator production than the serotype 2 strain P1/7 and the serotype 14 strain 13730²¹. However, the serotype 9 strain 1135776 used in the study was genetically distinct from the serotype 2 strain P1/7, suggesting that combination of CPS and genetic background of other factors, such as cell wall components, are important for virulence. Meanwhile, serotype switch to CPS7 or CPS3 has restricted impact and affected few of the evaluated parameters. The SS2to7 mutant has slightly increased susceptibility to killing by pig blood and reduced virulence in the swine infection model, being mainly recovered from tonsils. Interestingly, one of the SS2to7-infected pigs developed shivering, and bacteria were only recovered from the brain. It should be noted that serotype 7 strains are isolated in a greater proportion from the central nervous system than from other organs in diseased pigs³⁷. The SS2to3 mutant presented increased susceptibility to killing by mouse and pig blood, slightly reduced bacteremia in mice, and diminished capacity to induce cytokine production in vivo. Though serotype 3 CPS expression still caused *S. suis*-induced host death, clinical signs were less severe than those caused by SS2 in the mouse model. None of the pigs infected with SS2 developed clinical signs in experiment II, so a reduced virulence of SS2to3 mutant could not be definitively confirmed in the natural host. Overall, results obtained with the different mutants confirmed the delicate balance between bacterial burden, systemic dissemination, level of the inflammatory response, and clinical outcome^{32,38,39}. Given that only different CPSs were expressed between mutants, these differences in effects depending on switched serotypes might be due to differential cell wall component exposure, including adhesins and immunostimulatory components, and/or recognition of certain motifs of specific *S. suis* CPSs by unknown host cell receptors.

This work also highlighted the complexity of *S. suis* host–pathogen interactions and the carefulness required when analyzing data from single cell type cultures vs. more complex biological systems (such as blood). For

instance, neutrophils and monocytes are the main phagocytes in blood, with little to no macrophages being present. Therefore, results obtained with macrophages might not necessarily reflect *S. suis* fitness in blood, but rather mimic the situation in tissues. Similarly, the interactions of *S. suis* with swine blood leukocytes are more complex than those evaluated when using mouse blood due to the presence of swine antibodies reacting against the bacteria. Thus, by using multiple *in vitro* and *in vivo* models, a more comprehensive analysis is obtained.

In *Streptococcus pneumoniae*, strict evaluations of the CPS effects using CPS switch mutants have already been performed, and several studies demonstrated that capsule type affected resistance to both complement C3b deposition and opsonophagocytic uptake⁴⁰, nonopsonic neutrophil-mediated killing⁴¹, and adhesion to the pharyngeal or lung epithelial cells⁴². Some of these studies also indicated the effect on virulence within the respiratory tract⁴², colonization⁴¹, survival in blood⁴⁰, and brain injury⁴³ by *in vivo* infection models. The structure and composition of CPS8 of *S. suis* is known to be identical to that of *S. pneumoniae* serotype 19F¹⁶, with serotype 19F pneumococcus mutant being shown to be the most resistant to non-opsonic killing by human neutrophils among the mutants⁴¹, suggesting that this structure of CPS provides the bacteria with high resistance to killing in blood. Previous studies using serotype-switched mutants^{41,44} also showed that CPS type affects the degree of encapsulation and growth phenotype due to the difference in metabolic costs for producing capsule between CPS types. In one of the study⁴⁴, mix of the bacterial cells with thick capsule and thinner capsule was observed when the pneumococcus mutant switching CPS to serotype 19F was grown in the nutrient-limiting condition, unlike the other serotype-switched mutants (switching to serotypes 7F, 18C and 6B). These points should be evaluated in *S. suis*, especially serotype 8 CPS, in the future, because these may be one of the explanations of the difference in host interactions and virulence between SS2 and the serotype-switched mutants, in case different effect on the degree of encapsulation or mix of bacterial cells with thin and thick capsule, similar to the pneumococcus mutant switching CPS to serotype 19F, in the nutrient-limiting condition like *in vivo*.

In conclusion, these data demonstrate that serotype switching in *S. suis* serotype 2 can modulate host cell interactions and virulence. Among the tested serotypes, switch to serotype 8 increased the virulence. Although it remains unknown whether *S. suis* serotype switching affects virulence in humans, one serotype 8 strain having a genetic background similar to virulent serotype 2 clinical isolates has already been recovered (unknown source: pubMLST: <http://pubmlst.org/ssuis/>). Therefore, these results clearly demonstrate that more attention should be given to serotype switching in *S. suis* with regards to both commensal and pathogenic strains.

Methods

S. suis culturing. The *S. suis* strains used in this study are listed in Table 1. The serotype 2 strain P1/7 (SS2 in this study)²⁴ was used as the parental strain for construction of the serotype-switched mutants. P1/7 belongs to CC1 and was shown to be induced to a competent state using XIP¹⁸. *S. suis* strains of serotypes 3, 4, 7, 8, 9, and 14 were used as donors to construct the serotype-switched mutants. All strains were cultured overnight on Todd-Hewitt (TH) agar (Becton Dickinson, Franklin Lakes, NJ, USA) at 37 °C with 5% CO₂ unless indicated otherwise. Chloramphenicol was added to the medium at 5 µg/mL, when needed.

General molecular biology techniques. All PCRs were completed using the iProof HF Master Mix (Bio-Rad Laboratories, Hercules, CA, USA) and QIAGEN Multiplex Master PCR Mix (Qiagen, Hilden, Germany) according to the manufacturers' instructions. The PCR primers used in this study are listed in Supplementary Table S7. The amplified PCR products were purified using the QIAQuick PCR Purification Kit (Qiagen) and sequenced on a 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) where required. The sequence assembly of the PCR products was performed using SEQUENCHER 5.4 (Gene Codes Corp., Ann Arbor, MI, USA).

Construction of serotype-switched mutants and non-encapsulated mutant. An outline of the approach developed for the construction of the serotype-switched mutants is represented in Supplementary Fig. S1. First, a non-encapsulated mutant whose *cps* locus was replaced with a chloramphenicol resistance gene (Δ CPS2tocat) was generated from SS2. DNA fragments comprising the chloramphenicol cassette flanked by approximately 1 kbp of the upstream and downstream regions of the *cps* gene cluster were amplified by overlap-extension PCR. SS2 corresponding locus tags of genes deleted were SSU0515–SSU0538. The plasmid pSET1⁴⁵ was used as a template for the PCR to amplify the *cat* cassette. Five-microliters of the DNA fragment (approximately 500 ng) was then transformed into 100 µL of the SS2 culture [optical density 600 nm (OD₆₀₀) of 0.035–0.045] by inducing competent state with 10 µL of 2.5 mM XIP as previously described¹⁸. After selection of transformants by culturing on TH plates with chloramphenicol, non-encapsulation was confirmed by co-agglutination with anti-serotype 2 serum (Supplementary Fig. S1, panel 1). Then, 100 µL of the Δ CPS2tocat (OD₆₀₀ of 0.035–0.045) was transformed with 5 µL of whole genome of donor strains (approximately 2 µg of each genome DNA) and XIP (Fig. S1, panel 2). For screening the desired serotype-switched mutant candidates, bacterial cells were collected from the transformed culture by centrifugation at 2,600 × g for 20 min and washed once with 1 mL of 0.15 M NaCl. Percoll density gradient centrifugation was performed as previously described⁴⁶. The washed cells were suspended with 10 µL of the undiluted Percoll PLUS (GE Healthcare UK Ltd., Buckinghamshire, UK), and the bacterial cell suspension was added to the bottom of a 2 mL microtube. Four hundred microliters of 20, 40, 60, and 80% stock isotonic Percoll (SIP) solution was gently layered onto the washed cell suspension to produce a step gradient with 80% SIP at the bottom of the tube and 20% SIP at the top. The tube was centrifuged at 2600 × g for 20 min to separate bacterial cells according to density. After centrifugation, 100 µL of the solution was collected from the interface between 20 and 40% SIP and between 40 and 60%, since most of the *S. suis* cells considered to be non-encapsulated are concentrated at the interface between 60 and 80% SIP and those considered to be encapsulated at the interface between 20 and 40% and between 40 and 60%. The

collected solutions were spread and cultured on TH agar (Supplementary Fig. S1, panel 3). All of the colonies grown on the agars (100–200 colonies) were subcultured overnight at 37°C in sterile U-bottom 96-well plates (Corning, NY, USA) with 100 µL of TH broth. The cultures that formed clear precipitates at the tip sections of the bottoms were selected and subcultured overnight at 37°C with 5% CO₂ on both TH agar plates with and without chloramphenicol (Supplementary Fig. S1, panel 4). The cultures that grew only on TH agar without CP were chosen, and the gain of *cps* gene cluster from the introduced genome DNA was verified by *cps* type-specific PCR as previously described⁴⁵. Serotype switch was also confirmed using co-agglutination with antisera of the respective serotypes. For generation of the markerless non-encapsulated mutant, blue-white screening method using 5-bromo-4-chloro-3-indoxyl- α -L-fucopyranoside (X- α -L-fucopyranoside) was performed as represented in Supplementary Fig. S2 (See Supplementary Methods for more detail).

S. suis growth measurements. Strains were streaked onto TH agar plates and incubated overnight at 37 °C with 5% CO₂ and then subcultured in TH broth to OD₆₀₀ of 0.6 using a spectrophotometer Ultrospec 2100 (Biochrom Ltd., Cambridge, UK). After adding 1/500 of the volume of each adjusted culture diluted 1,000 times by TH broth to TH broth, the cultures were incubated at 37 °C under air plus 5% CO₂ conditions. The CFU (/mL) of each of the cultures was measured at 2, 4, 6, 8, 10, 12, and 14 h after incubation by plating serial dilutions on TH agar.

Confirmation of serotype switching. Serotyping, cell surface hydrophobicity test, TEM, measurement of CPS yields, NMR spectroscopy were performed to confirm well-encapsulation and serotype switching as previously described [22,47, serotyping and TEM; 27, hydrophobicity tests; 12,14–16, CPS purification and NMR] (see Supplementary Methods for more detail).

Whole genome sequence analyses. Whole genome draft sequences were determined using Illumina HiSeq X ten sequencing platform at the Beijing Genomics Institute (Shenzhen, China) or Illumina NovaSeq platform at Novogene Corporation (San Diego, CA, USA) (See Supplementary Methods for more detail). The final draft genome sequence of each of the mutants was then mapped and aligned with the publicly available complete genome sequence of strain P1/7 using Geneious Prime ver. 2019.1.1 (Tomy Digital Biology, Tokyo, Japan) with the default parameters. Calculations of ANI and a fraction shared between genome pairs were conducted using FastANI⁴⁸.

In vitro assays for evaluation of impacts on serotype switching. Adhesion and invasion assays using the porcine tracheal epithelial NPTr cell line, phagocytosis assays using J774A.1 murine macrophages, murine whole blood bactericidal assay using blood collected from 6- to 10-week-old C57BL/6J mice and from a five-week-old piglet, and measurement of pro-inflammatory mediator production by DCs generated using the femur and tibia of C57BL/6J mice were performed as previously described^{21,32,49}. (see Supplementary Methods for more detail).

In vivo assays for evaluation of impacts on serotype switching. Mouse infections were performed using 10–12 six-week-old male and female C57BL/6J mice per group via intraperitoneal inoculation (dose of 1×10^7 CFU/mouse) for survival and blood bacterial burden evaluation as previously described³². Plasma (systemic) pro-inflammatory mediators were measured using blood collected from eight mice intraperitoneally infected with 1×10^7 CFU 12 h post-infection as previously described³². Pig infections were performed for evaluation of appearance of symptoms and organ dissemination using 4–5 five-week-old crossbred male and female piglets per group purchased from Shokukanken Inc. (Gunma, Japan) or CIMCO Co. Ltd. (Tokyo, Japan). Infections were carried out via intranasal inoculation (dose of 2×10^9 CFU) for survival as previously described⁵⁰ and divided into two experiments per four groups (Experiment I: SS2, Δ CPS2, SS2to4, and SS2to7; experiment II: SS2, SS2to3, SS2to8, and SS2to14) (see Supplementary Methods for more detail).

Statistical analyses. Normality of data distribution was verified using the Shapiro–Wilk test and Mann–Whitney rank sum tests were performed to evaluate statistical differences between groups. Data are presented as mean \pm SEM or as geometric mean. Log-rank (Mantel–Cox) tests were used to compare survival between groups of mice. $P < 0.05$ was considered statistically significant.

Ethics statement. The animal experiments in this study were carried out in compliance with the ARRIVE guidelines and approved by the institutional committees for Ethics of Animal Experiments of the National Institute of Animal Health Japan (approval numbers 17-002, 17-010, and 17-085) and by the Animal Welfare Committee of the University of Montreal (approval number Rech-1570). Both committees formulated the guidelines and policies required to meet and adhere to the standards in the Guide for the Care and Use of Laboratory Animals.

Data availability

The sequence assembly data determined in this study and their raw data files were deposited in the DDBJ/ENA/GenBank databases under the accession numbers (P1/7, WABV00000000 and SRR13496243; Δ CPS2to4, WABW00000000 and SRR13496636; SS2to3, WABX00000000 and SRR13488957; SS2to4, WABY00000000 and SRR13488797; SS2to7, WABZ00000000 and SRR13489086; SS2to8, WACA00000000 and SRR13489169; SS2to9,

JABMDA00000000 and SRR13485874; SS2to14, WACB00000000 and SRR13489049; MO690, WACC00000000 and SRR13515771; MO691, WACD00000000 and SRR13516280; MO941, WACE00000000 and SRR13516281).

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Author contributions

M.Okura wrote and revised the manuscript, conceived, designed and conducted the experiments, analyzed that data. J.P.A wrote and revised the manuscript, designed and conducted the experiments, analyzed that data. T.S., G.G.D, M.R.V.C., F.M., and M.K. revised the manuscript, designed and conducted the experiments, analyzed that data. M.Osaki revised the manuscript and helped to analyze the data. M.S., M.G., and D.T. revised the manuscript and conceived the work.

Competing interests

The authors declare no competing interests.

Additional information

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