Loss of capsule among *Streptococcus suis* isolates from porcine endocarditis and its biological significance

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Streptococcus suis, particularly serotype 2, is a pathogen of both pigs and humans associated with a wide range of diseases, including meningitis, septicaemia and endocarditis. Among the genes in the capsular polysaccharide biosynthesis (*cps*) locus, *cps2J* exists only in the serotype 2 and 1/2 strains; therefore, *cps2J*-positive strains are suspected to have capsules of serotype 2 or 1/2. Coagglutination using antiserotype 1 and antiserotype 2 sera and/or transmission electron microscopy analysis of 288 *cps2J*-positive isolates from pigs showed that 32 (100 %) isolates from meningitis were encapsulated, whereas 86 (34 %) of 256 isolates from endocarditis were unencapsulated, indicating that capsule loss often occurred in the isolates from endocarditis. To investigate the genetic backgrounds, we randomly selected 43 unencapsulated isolates and analysed their *cps* loci by PCR scanning. Among them, 8 and 10 isolates apparently had deletions and insertions, respectively, in *cps* loci. In addition, a representative unencapsulated isolate and an unencapsulated strain showed adherence to porcine and human platelets, a major virulence determinant for infective endocarditis, to a significantly greater extent than the encapsulated strains. Although the capsule is considered to be an important virulence factor in *S. suis*, these results suggest that loss of capsular production is beneficial to *S. suis* in the course of infective endocarditis.

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INTRODUCTION

Streptococcus suis is a zoonotic pathogen that causes serious diseases, including meningitis, arthritis, septicaemia and

endocarditis, in swine and humans (Gottschalk *et al.*, 2007). *S. suis* strains are classified into more than 30 serotypes according to the different antigenicity of their capsular polysaccharides (Higgins & Gottschalk, 2006). Among them, serotype 2 has been predominantly isolated from both infected pigs and human patients in many countries (Higgins & Gottschalk, 2006; Wertheim *et al.*, 2009). Production of *S. suis* capsule is mediated by capsular polysaccharide biosynthesis (*cps*) genes clustered in a single locus of the genome. Among the genes in the *cps* loci identified to date (Holden *et al.*, 2009; Smith *et al.*, 1999a, b, c, 2000), *cps2J* has been found only in strains of serotypes 2 and 1/2 (the serotype reacting with both antisera 1 and 2 sera); therefore, this gene is sometimes used as a molecular marker of the two serotypes (Smith *et al.*, 1999c). However, for determination of the serotypes, it is necessary to verify their phenotypes by serotype-specific antisera.

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Abbreviations: CcpA, catabolite control protein A; GBS, group B *Streptococcus*; IS, insertion sequence; TEM, transmission electron microscopy.

The GenBank/EMBL/DDBJ accession numbers for the nucleotide sequence data reported in this paper are AB627103, AB627104, AB627105, AB627106, AB627107, AB627108, AB627109, AB627110, AB627111 and AB627112 for the inserted sequences of *S. suis* NL85, NL155, NL171, NL176, NL179, NL184, NL194, NL198, NL201 and NL255, respectively.

Two supplementary tables are available with the online version of this paper.

Endocarditis caused by *S. suis* is often found in adult pigs, particularly in slaughterhouses. In Japan, most of the endocarditis isolates are *cps2J* positive by PCR (our unpublished observation), suggesting that they are serotype 2 or 1/2. However, more than 50 % of the *S. suis* isolates from porcine endocarditis were shown to be untypable by agglutination tests (Katsumi *et al.*, 1997), although a high percentage of isolates from meningitis and pneumonia were serotypable (Kataoka *et al.*, 1993). Although the polysaccharide capsule is believed to be essential for the virulence of *S. suis* (Benga *et al.*, 2008; Chabot-Roy *et al.*, 2006; Charland *et al.*, 1998; Smith *et al.*, 1999a), these observations imply that many endocarditis isolates, especially those of serotypes 2 and 1/2, frequently lose their ability to synthesize capsules.

To confirm this speculation, we investigated the capsule production of *cps2J*-positive isolates from porcine endocarditis and meningitis. Furthermore, the genetic backgrounds of several unencapsulated isolates and the biological significance of unencapsulation were examined. Here we show that capsule loss often occurred in *cps2J*-positive endocarditis isolates and that unencapsulation increased the ability of the bacteria to adhere to platelets, which is thought to be a major virulence determinant in the pathogenesis of infective endocarditis.

METHODS

Bacterial strains and growth conditions. A total of 288 *cps2J*-positive *S. suis* isolates from different pigs were used in this study. Among them, 256 isolates were from heart valve vegetations of pigs with endocarditis in regional diagnostic centres in Japan between 1994 and 2009, and 32 isolates, including the well-characterized strain P1/7 (Slater *et al.*, 2003), were from pigs with meningitis. Except P1/7, all of the meningitis isolates were isolated in regional diagnostic centres or the National Institute of Animal Health in Japan between 1989 and 2006. All isolates were stored in Luria–Bertani (Becton Dickinson) broth containing 30 % glycerol at -80°C and minimally passaged for the experiments to avoid changing key traits including capsule production. In addition, *S. suis* strains S735 (NCTC 10234; serotype 2 reference strain) and 204 (serotype 1 field isolate) (Sekizaki *et al.*, 2001) were used for the production of rabbit antiserum, and *S. suis* strain 89/1591, isolated from a pig with septicaemia (Salasia *et al.*, 1995), and its isogenic unencapsulated mutant (CPS2B) (Okura *et al.*, 2011) were used to compare their phenotypic characteristics with other encapsulated and unencapsulated *cps2J*-positive isolates. *Enterococcus faecalis* NCTC 775 was used as a control for PCR scanning analysis. Identification of *S. suis* field isolates was confirmed by species-specific PCR for *S. suis* (Okwumabua *et al.*, 2003) and/or sequencing of the 16S rRNA gene. The presence of *cps2J* was examined by PCR as described previously (Silva *et al.*, 2006). Bacteria were cultured in Todd–Hewitt broth (THB; Difco Laboratories, Becton Dickinson) or agar (THA) at 37°C in air plus 5 % CO_2 for 16 h, unless otherwise indicated. For strain CPS2B, of which the *cps2B* gene was disrupted by the insertion of a suicide vector containing a spectinomycin resistance gene, spectinomycin ($100\text{ }\mu\text{g ml}^{-1}$) was added to the medium.

Production of rabbit antisera. Rabbit antiserotype 1 and antiserotype 2 polyclonal sera were prepared by immunizing rabbits with formalin-killed *S. suis* strains 204 and S735, respectively,

according to the procedure of Higgins & Gottschalk (1990). Briefly, rabbits weighing 3 kg were given three injections per week of increasing numbers of bacteria for 4 weeks as follows: first week, $2\text{--}4 \times 10^9$ c.f.u.; second to fourth week, $4\text{--}8 \times 10^9$ c.f.u.. Ten days after the last injection, blood samples were collected and the sera were evaluated by coagglutination tests as described below. All animal procedures were carried out according to the regulations and guidelines approved by the Animal Ethics Committee of the National Institute of Animal Health.

Serotyping. The capsular antigens of all *cps2J*-positive isolates cultured on THA plates were extracted by autoclaving the cells in Dulbecco's PBS (DPBS) at 121°C for 15 min and tested with antiserotype 1 and 2 sera. The coagglutination technique was applied as previously described (Gottschalk *et al.*, 1989; Han *et al.*, 2001). The reaction was judged as positive when agglutination occurred within 5 min. For isolates with a negative agglutination reaction, further coagglutination tests were performed to verify the absence of capsule using isolates subcultured under the conditions recommended to enhance capsular production (Gottschalk *et al.*, 1993).

Transmission electron microscopy (TEM). The samples were prepared according to previous studies (Jacques *et al.*, 1990; Mackie *et al.*, 1979) with some modifications. Briefly, bacterial cells were harvested from cultures on THA plates, washed with PBS (0.01 M, pH 7.2), and incubated with antiserotype 2 serum at 4°C for 1 h. The cells were then washed with deionized distilled water (DDW) and fixed with 5 % (v/v) glutaraldehyde containing 0.15 % (w/v) ruthenium red at room temperature for 2 h. The cells were immobilized in 1 % agar, post-fixed with 1 % osmium tetroxide at 4°C for 1.5 h, and washed once with DDW. Samples were then dehydrated with a graded series of ethanol and embedded in low-viscosity resin (Quetol 651 mixture; Nisshin EM). Ultrathin sections were stained with uranyl acetate and lead citrate prior to examination with a transmission electron microscope (H-7500; Hitachi).

PCR scanning of genes in the *cps* loci and sequencing of mutated regions. Twenty-one primer sets covering the whole *cps* locus of serotype 2 (Table 1, Fig. 1) were designed on the basis of the *S. suis* strain P1/7 genome sequence data (accession number AM946016). Chromosomal DNA extracted by standard procedures (Mogollon *et al.*, 1990) was used as template DNA, and PCRs were performed using *Ex Taq* polymerase (Takara Bio) according to the manufacturer's instructions. The conditions of the PCR scanning assay consisted of pre-denaturing at 95°C for 5 min, 30 cycles of 20 s at 95°C , 10 s at 55°C , 1 min at 72°C , and final extension at 72°C for 2 min. Chromosomal DNA of *S. suis* P1/7 and *E. faecalis* NCTC 775 was used as positive and negative controls, respectively. PCR products were analysed by electrophoresis on 1 % agarose gel and/or a MultiNA microchip electrophoresis system (Shimadzu Biotech).

The PCR products of sizes different from those of *S. suis* P1/7 were purified by a QIAquick PCR purification kit (Qiagen) following the manufacturer's instructions and sequenced by primer walking. Additional PCR and inverse PCR (Ochman *et al.*, 1988) using different combinations of primers were performed to amplify the altered *cps* regions. The PCR products were sequenced by a BigDye terminator v3.1 cycle sequencing kit using a 3130xl Genetic Analyzer (Applied Biosystems). Sequencher Ver. 4.8 (Hitachi Software Engineering) and Artemis software (Wellcome Trust Sanger Institute, <http://www.sanger.ac.uk>) were used for assembly and analysis of the sequences. Deletions and insertions were determined using the CLUSTAL W Ver. 1.83 (<http://clustalw.ddbj.nig.ac.jp/top-e.html>) and BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) programs. In addition, IS Finder (<http://www-is.biotoul.fr/>) was used to annotate and classify insertion sequence (IS) elements.

Table 1. Primer sets for PCR scanning

Reaction no.	Forward	Sequence (5'-3')	Reverse	Sequence (5'-3')	Product size in strain P1/7 (bp)
1	cps2-F1	AGGTAAAAGTCTAGGAAGGT	cps2-R1	ACGGACTGAATCGCTGCCTT	1455
2	cps2-F2	TTAATCGACTTGGTGGGTGG	cps2-R2	GAAGAAACCAGGCATGACTG	1451
3	cps2-F3	AGCGGAAGAACCAACCACTC	cps2-R3	CGCCAATTAAGCAGGCTTC	1441
4	cps2-F4	CGAAGCTTATCGTCAAGGTG	cps2-R4	TCATCAAGATGTGACTAGGC	1472
5	cps2-F5	CGTGGTGCCGTGTATTTAC	cps2-R5	CATGACCGTCTGGGTTTACG	1520
6	cps2-F6	GGACCATCTGGTCAGACATT	cps2-R6	CCTACGTAGAATTGGATAGT	1476
7	cps2-F7	GGAACGAACCCATCTTTACT	cps2-R7	ACTTTGAGGGAGGTGTAGAC	1412
8	cps2-F8	GAGTGGCGAGTAGTAGAATT	cps2-R8	CGATATTGTTCTCCATAGTAGC	1490
9	cps2-F9	GAGGCATATAATCAGTATCG	cps2-R9	TTGCTGACATATTCGATAG	1492
10	cps2-F10	GGTACAGGTGTAGACTTGTC	cps2-R10	GTTGATTCCATAGTAGATATAG	1422
11	cps2-F11	TGATTCTTACGCTCATCGCG	cps2-R11	AGAGGACGTTTCGTTAATAC	1480
12	cps2-F12	AAGAGGTGCGAGACTTAGGA	cps2-R12	AAGCTTCTTTTGCTGTTTGC	1398
13	cps2-F13	TCAGGCTGTCTGAGCGGCA	cps2-R13	ATTGCTGGAATCTTCTGTCC	1449
14	cps2-F14	ATCGGGTCCAGGGAGTTGGG	cps2-R14	GGATCTGATACATCGTATGG	1464
15	cps2-F15	TCAGAAATGTATAAGGGGGG	cps2-R15	GTTCCAATCGTATAGACGAG	1481
16	cps2-F16	AGCAAGGGCGATAGTAGCGG	cps2-R16	CAGATAGGAAGCAGTCGTTG	1566
17	cps2-F17	TGGGCTTCATTTTCGAAGG	cps2-R17	GGTATTCTGCCTTTGGTGCG	1502
18	cps2-F18	CTCTACCATGAAAATTGTGC	cps2-R18	ATCCTACGCTTGTCGCGTTC	1552
19	cps2-F19	CTGGACAATGAAATGGAAGG	cps2-R19	CATGGTTGAGGCCTGTACAG	1466
20	cps2-F20	GGAGGATAACACAGCCGAAG	cps2-R20	CTAACAACGTGGTCAGCTTG	1533
21	cps2-F21	CAGTGATTCAATGTATCGAG	cps2-R21	CCAACATAGTCTAGCCACTG	1400

Bacterial adherence to porcine and human platelets. The ability of encapsulated and unencapsulated strains to adhere to porcine and human platelets was evaluated according to a previous study (Hoshino *et al.*, 2009) with appropriate modifications. For preparation of the inocula, *S. suis* strains were cultured in THB or THB with 100 µg spectinomycin ml⁻¹ (for strain CPS2B) until the OD₆₀₀ reached 0.8. The cultured bacteria were washed twice with DPBS, sonicated in DPBS for 30 s to disperse the bacterial cells, and then diluted with DPBS to approximately 2 × 10⁹ c.f.u. ml⁻¹. The bacterial suspensions were additionally diluted in triplicate and each dilution was plated twice onto THA to examine the exact concentration of the inocula each time.

Porcine venous blood was freshly obtained from healthy adult pigs kept in the National Institute of Animal Health, and the platelets were prepared by centrifuging the blood at 100 g for 15 min and collecting the upper layer. Human platelets donated for transfusion were obtained from the Japan Red Cross Society. The collected platelets were washed twice with platelet wash buffer [0.14 M NaCl, 20 mM HEPES, 1 mM EDTA (pH 6.6) containing 1 µg prostaglandin I₂ ml⁻¹ for the first wash], fixed with 0.8 % formalin in DPBS, and immobilized in eight-well culture slides (BD Falcon glass; Becton Dickinson) coated with 0.01 % poly-L-lysine solution at approximately 1 × 10⁸ platelets per well. The wells were treated with

1 × blocking reagent (Roche Applied Science) for 1 h with gentle rocking at room temperature to minimize non-specific adherence. After removal of the blocking reagent by aspiration, 500 µl of each bacterial suspension was inoculated into the wells to give an m.o.i. of approximately 10. After incubation with gentle shaking for 2 h at room temperature, platelets were washed four times with DPBS to remove the unattached bacteria, fixed with pre-chilled methanol, and stained with 5 % Giemsa solution. The numbers of bacterial cells attached to 100 platelets were determined by light microscopy. In each strain/isolate, the assay was repeated six times using porcine platelets from two different pigs and twelve times using human platelets of three different lots. The differences were analysed by Student's unpaired *t*-test at 95 % confidence interval (*P* < 0.05).

RESULTS

Loss of capsule in *cps2J*-positive endocarditis isolates

A total of 288 *cps2J*-positive isolates were examined for capsular production by the coagglutination test. All 32

Fig. 1. Genetic organization of the serotype 2 *cps* locus and positions of primers used for PCR scanning. Grey arrows represent genes putatively involved in capsule synthesis. Black arrows indicate genes encoding hypothetical proteins. White arrows indicate pseudogenes. White and black arrowheads represent forward and reverse primers, respectively.

isolates from meningitis exhibited agglutination with antiserotype 2 serum, and 4 were also agglutinated with antiserotype 1 serum, indicating that the 28 and 4 isolates were serotypes 2 and 1/2, respectively. In contrast, although 170 (66 %) isolates from endocarditis were agglutinated with antiserotype 2 serum, 86 (34 %) isolates were not agglutinated with either antiserotype 1 or antiserotype 2 sera, suggesting that these isolates have lost their capsule. To confirm the results, we selected two coagglutination-positive (P1/7 and NL333) and four coagglutination-negative (NL146, NL194, NL240 and NL290) isolates and examined them by TEM. As shown in Fig. 2, cells of both coagglutination-positive isolates were surrounded by a thick capsule, whereas no capsular material could be seen in the coagglutination-negative isolates. These results demonstrate that, unlike meningitis isolates, notable numbers of endocarditis isolates lose their ability to produce the capsule.

Structural alterations have occurred in the *cps* loci of the unencapsulated isolates

To investigate the genetic backgrounds affecting capsular expression, we randomly selected 43 representative unencapsulated isolates and analysed the genes in their *cps* loci by PCR scanning. As shown in Supplementary Table S1, available with the online version of this paper, 18 isolates gave no and/or unexpected amplifications with at least one of the primer sets, indicating structural alterations in the *cps* loci. Nucleotide sequencing of those regions showed that genes in the *cps* loci of 8 and 10 isolates had deletions and insertions, respectively (Fig. 3). However, for the other 25 isolates, no apparent alteration was found in their *cps* regions by PCR scanning.

Three isolates (NL157, NL191 and NL280) had a partial deletion in the *neuB*, *cps2E* and *cps2G* genes, respectively, whereas five (NL204, NL217, NL268, NL290 and NL319)

had a deletion of multiple genes, as shown in Fig. 3. Among the five isolates above, a large deletion (approx. 17.5 kb fragment including the *neuB*–*A* genes) was found in NL217. In addition, this isolate had a 192 bp deletion in the *cps2F* gene. In NL204 and NL290, >3 kb deletions were found in almost the same region (*cps2A*–*E* genes), while the SSU0514–*cps2A* and *cps2F*–*G* regions were lost in NL268 and NL319, respectively.

IS or putative IS elements also disrupted *cps* genes (Fig. 3, Supplementary Table S2). The IS elements found in NL85 and NL194 were classified into the ISL3 family, while those of NL155, NL171, NL198, NL201 and NL255 were classified into the IS110 family. Although a putative IS element found in NL184 did not show significant similarity to any other known IS elements, the closest relative was ISTel1 of *Thermosynechococcus elongatus* BP-1, which belongs to the IS481 family (Supplementary Table S2). However, the sequences inserted in the *cps* loci of NL176 and NL179 encoded putative reverse transcriptases that constitute group II introns (Michel & Ferat, 1995).

Notably, most of the deletions and insertions were found in the *cps2A*–*G* and *neuB*–*A* regions. In particular, *cps2E* was a hot spot for mutations, as 7 of 10 insertions were found in this gene. On the other hand, the *cps2H*–SSU0534 region of the isolates analysed in this study was rarely affected.

Unencapsulation increases the ability of *S. suis* to adhere to porcine and human platelets

Adherence of bacteria in the bloodstream to platelets on the damaged endocardial surface is thought to be an important mechanism for the initial colonization of cardiac valves (Sullam *et al.*, 1996). To investigate the biological significance of the unencapsulation of *S. suis* in the pathogenesis of infective endocarditis, we compared the ability of encapsulated strain P1/7 and unencapsulated

Fig. 2. Transmission electron micrographs of ultrathin sections of *S. suis* strains. Cells of strains P1/7 (a) and NL333 (b) were obviously surrounded by the capsule, while capsular materials were not seen in strains NL146 (c), NL194 (d), NL240 (e) and NL290 (f). Bars, 0.5 μ m.

Fig. 3. Positions and types of structural alteration in the *cps2* loci of representative unencapsulated endocarditis isolates. Arrowheads indicate positions of insertion. Grey lines indicate deleted regions. Grey arrows represent genes putatively involved in capsule synthesis. Black arrows indicate genes encoding hypothetical proteins. White arrows indicate pseudogenes.

endocarditis isolate NL194 to adhere to porcine platelets. As shown in Fig. 4(a, b), the mean number of attached bacteria per porcine platelet of NL194 (2.55 ± 0.3) was significantly higher than that of P1/7 (0.21 ± 0.07) ($P < 0.05$). To evaluate the effect of unencapsulation more precisely, we further analysed the encapsulated serotype 2 strain 89/1591 and its isogenic unencapsulated mutant CPS2B. CPS2B adhered to porcine platelets in greater numbers than 89/1591 (mean no. of attached bacteria per porcine platelet: 2.02 ± 0.2 for CPS2B vs 0.44 ± 0.15 for 89/1591, $P < 0.05$) (Fig. 4a, b). Similar results were obtained when human platelets were used (mean no. of attached bacteria per human platelet: 2.16 ± 0.22 for NL194 vs 0.53 ± 0.38 for P1/7, $P < 0.05$; and 2.2 ± 0.37 for CPS2B vs 0.92 ± 0.7 for 89/1591, $P < 0.05$) (Fig. 4c). These results suggest that unencapsulation facilitates the adherence of *S. suis* to platelets in both swine and humans.

DISCUSSION

In addition to the molecular basis for serotyping in *S. suis* (Higgins & Gottschalk, 2006), the polysaccharide capsule has been shown to be an important virulence factor of this pathogen. Compared with the parent strains, the isogenic unencapsulated mutants of *S. suis* serotype 2 were more susceptible to phagocytosis by both macrophages and neutrophils (Benga *et al.*, 2008; Chabot-Roy *et al.*, 2006; Charland *et al.*, 1998; Smith *et al.*, 1999a). Moreover, capsule loss reduced the virulence of *S. suis* in both mouse and swine models of infection (Charland *et al.*, 1998; Smith

et al., 1999a). In contrast, Salasia *et al.* (1995) and Benga *et al.* (2004, 2005) reported that isogenic unencapsulated mutants showed increased adherence to various types of cells, including porcine endothelial cells and human epithelial cells, when compared with the parent serotype 2 strains. Esgleas *et al.* (2005) also demonstrated that an unencapsulated mutant bound to extracellular matrix proteins to a higher degree than its parental encapsulated serotype 2 strain.

In this study, we demonstrated capsule loss in a notable number of *S. suis* isolates from pigs with endocarditis. In agreement with previous studies, the unencapsulated mutants showed a higher degree of adherence to porcine platelets than the encapsulated strains. Bacterial adherence to platelets is considered to be a major virulence determinant in the pathogenesis of infective endocarditis (Sullam *et al.*, 1996). In association with prior injury or disease of the heart valves, the endothelial or exposed connective tissue surface becomes coated with platelets and fibrin. Subsequently, the circulating micro-organisms adhere to and colonize the platelet vegetation, and the colonies are typically encased in more platelets and fibrin, forming the primary infectious lesion or septic vegetation (Herzberg, 1996). Thus, the frequent isolation of unencapsulated isolates from porcine endocarditis might result from the enhanced ability of these isolates to form septic vegetations.

Recently, Tanabe *et al.* (2010) reported that a capsular-deficient *S. suis* mutant had acquired the capacity to form a thick biofilm, which was not observed in the parent strain.

Fig. 4. Bacterial adherence to porcine and human platelets. (a) Adherence of encapsulated and unencapsulated *S. suis* strains/isolate to porcine platelets. Bacterial cells and platelets were stained with 5% Giemsa solution and observed by light microscopy: A, encapsulated serotype 2 strain P1/7; B, unencapsulated endocarditis isolate NL194; C, encapsulated serotype 2 strain 89/1591; D, isogenic unencapsulated mutant of strain 89/1591 (CPS2B). Black and white arrowheads indicate platelets and bacterial cells, respectively. Images taken at $\times 1000$ magnification. (b, c) Ability of *S. suis* strains and isolate NL194 to adhere to porcine (b) and human (c) platelets. Asterisks indicate significant differences ($P < 0.05$).

Although the exact role of biofilm formation in *S. suis* infections is unclear, such a property may allow the bacteria to become persistent colonizers and to resist clearance by the host immune system. The biofilm-positive phenotype of unencapsulated mutants therefore might give them further advantage in forming cardiac vegetations in swine.

In this study, increased adherence of the unencapsulated isolates to human platelets was also observed. Although human cases of endocarditis caused by unencapsulated *S. suis* have not yet been reported, an unencapsulated isolate from the blood of an endocarditis patient has been described for group B *Streptococcus* (GBS) (Sellin *et al.*, 1992). Therefore, our results may suggest the potential of unencapsulated *S. suis* to cause infective endocarditis in humans.

Because encapsulated isolates were retrieved from 66% of the porcine endocarditis cases analysed in this study, even in cases from which unencapsulated *S. suis* were isolated, it is unknown whether all bacterial cells present in the vegetations were unencapsulated or whether only a subpopulation of infected bacteria had lost the capsule and the unencapsulated cells established a footing as the first colonizers for further colonization of encapsulated cells. It is also unclear whether unencapsulation occurred after invasion of the bloodstream by bacteria or whether

unencapsulated strains can be transmitted among different pigs and farms. Interestingly, we found five isolates (NL155, NL171, NL198, NL201 and NL255) with almost the same IS elements (99.8–100% identical at the nucleotide level) at the same position of *cps2E* (Fig. 3, Supplementary Table S2). Among them, NL155, NL171, NL198 and NL201 were isolated from different pigs in the same prefecture, while NL255 was isolated from the adjacent area. This strongly suggests the transmission of unencapsulated *S. suis* strains among pigs and farms, although we cannot rule out the possibility that the insertion position was the preferential site for the IS elements and insertion events occurred independently in each isolate. It is of note that most of the deletions and insertions found in the unencapsulated isolates occurred in the *cps2A–G* region. Among the genes in this region, *cps2E* was the most frequently altered. This may support the notion that *cps2E* and its flanking regions are hot spots for structural alteration.

No apparent deletion or insertion was detected by PCR scanning in the 25 unencapsulated isolates. During preparation of this paper, Willenborg *et al.* (2011) reported that catabolite control protein A (CcpA) of *S. suis* is necessary for capsular expression and that deletion of *ccpA* resulted in significant reduction of capsule thickness. However, the deduced amino acid sequences of *ccpA* of

the 25 unencapsulated isolates were 99.4–100 % identical to that of strain P1/7 and 99.7–100 % identical to that of strain 89/1591 (our unpublished observations), suggesting that *ccpA* of the unencapsulated isolates was intact. Therefore, their *cps* loci may be affected by point mutations that could not be detected by PCR scanning. Alternatively, it is also conceivable that their capsular expression was negatively regulated by unknown mechanisms. In fact, phase variation of capsular expression has previously been reported in a GBS isolate. In this case, although the original strain isolated from the blood of a patient with endocarditis was unencapsulated, encapsulated variants could be recovered after Percoll gradient centrifugation (Sellin *et al.*, 1992). Similar phase variations have been found in other Gram-positive and -negative bacteria (van der Woude & Bäumler, 2004). Although such variations have not been reported in *S. suis*, capsular expression might be reversible in some unencapsulated *S. suis* isolates. Moreover, even in unencapsulated isolates with mutations affecting capsular expression, capsule production could be restored by acquiring functional genes via horizontal gene transfer, additional point mutations or the excision of inserted sequences. Further studies to investigate the above possibilities will provide additional insights into the role of the capsule in the pathogenesis of *S. suis* infection.

It is noteworthy that the *cps* loci of NL176 and NL179 were similarly disrupted by the insertion of a putative group II intron within SSU0529. Because SSU0529 is considered to be a pseudogene, it cannot be assumed that the disruption of SSU0529 itself caused the loss of capsular production in these isolates. Thus, in these two isolates, additional point mutations and/or negative regulation of capsular expression might occur in addition to the insertion events. Alternatively, the expression of downstream genes might be influenced by a polar effect caused by insertion of the group II intron.

In conclusion, we have demonstrated that approximately one-third of the endocarditis isolates from swine that we examined had lost the ability to produce capsules and that unencapsulation enhanced bacterial adherence to porcine and human platelets. Although the capsule is thought to be an important virulence factor for *S. suis*, our results suggest that loss of capsule production may be beneficial for *S. suis* in causing infective endocarditis.

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