Doctorate Thesis

Title of Thesis Studies on the capsular polysaccharide synthesis genes in *Streptococcus suis*

（豚レンサ球菌の莢膜多糖合成遺伝子に関する研究）

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# General Introduction

*Streptococcus suis* (*S. suis*) is a Gram-positive coccus which commonly occurs as single cells, in pairs or in short chains (Wertheim *et al.*, 2009). *S. suis* can be naturally found in the upper respiratory tract, especially in tonsils and nasal cavities, genital and alimentary tracts including salivary glands of healthy pigs (Arends *et al.*, 1984; Gottschalk & Segura, 2000; Padungtod *et al.*, 2010). The rate of asymptomatic carrier in adult pigs may be nearly 100% but the morbidity rarely exceeds 5% (Clifton-Hadley *et al.*, 1986). Sow is a potential source of infection in the herd, and piglets can become infected from the sow harboring *S. suis* in uterus or vagina. However, the infection more likely occurs at weaning when they are moved, mixed and resided together (Staats *et al.*, 1997). The carriers are also important for transmission of *S. suis* in herds via nasal and oral routes (Lun *et al.*, 2007). The presumptive identification of *S. suis* is basically relied on the biochemical tests as following parameters: Voges-Proskauer negativity, hydrolysis of esculin positivity, trehalose positivity and negativity for growth in 6.5% NaCl (Tarradas *et al.*, 1994). The molecular techniques using polymerase chain reaction (PCR) on the basis of *S. suis*-specific glutamate dehydrogenase gene (*gdh*) (Okwumabua *et al.*, 2003), PCR on the basis of *S. suis*-specific 16s rRNA region and a species-specific probe targeting 16s rRNA gene (Chatellier *et al.*, 1998; Lun *et al.*, 2007) are reliable tools for identification of *S. suis*.

According to the different antigenicity of the capsular polysaccharides, *S. suis* strains can be classified into 35 serotypes (serotypes 1-34 and serotype 1/2, which reacts with both anti-serotypes 1 and 2 sera) (Higgins & Gottschalk, 2006) but serotypes 32 and 34 were latter demonstrated to be *Streptococcus orisratti* (Hill *et al.*, 2005). More recently, on the basis of the phylogenetic analyses of gene encoding the manganese-dependent superoxide dismutase (*sodA*) and a gene encoding recombination/repair protein (*recN*), serotypes 20, 22, 26 and 33 were suggested to be taxonomically removed from *S. suis* (Tien le *et al.*, 2013). The serotype identification method is generally based on the reaction between polysaccharide capsular

antigens and specific antiserum of each serotype using capsular reaction (Neufeld), capillary precipitation or co-agglutination test (Gottschalk *et al.*, 1989; Perch *et al.*, 1983). However, the serotyping on the basis of specific antiserum is laborious and costly. The multiplex PCR and PCR typing method using serotype-specific gene have been developed and at least 15 serotypes (serotypes 1, 2, 3, 4, 5, 7, 8, 9, 10, 14, 16, 19, 23, 25 and 1/2) can be effectively identified to date (Kerdsin *et al.*, 2009; Kerdsin *et al.*, 2012; Okwumabua *et al.*, 2003; Smith *et al.*, 1999b; Smith *et al.*, 1999c; Wang *et al.*, 2011; Wang *et al.*, 2012; Wisselink *et al.*, 2002).

Among all serotypes so far described, serotype 2 was predominantly isolated from both infected pigs and patients (Higgins & Gottschalk, 2006; Wertheim *et al.*, 2009). *S. suis* serotype 2 can tolerate in wide range environmental conditions. Although *S. suis* serotype 2 can easily be inactivated by 5% bleach at 1:799 dilution, this microorganism can survive at 60° C for 10 min, at 50° C for 2 h, and at 10° C for 6 weeks in carcasses (Clifton-Hadley & Enright, 1984). At 0° C, it can persist for 1 month in dust and 3 months in feces and at 25° C, it can live for 24 h in dust and 8 days in feces (Clifton-Hadley & Enright, 1984). Due to its tolerance, *S. suis* serotype 2 can persist in various environments and often causes infection in many countries.

In pigs, *S. suis* infection has been reported in many countries of North America (United States and Canada), South America (Brazil), Europe (United Kingdom, Belgium, Denmark, Norway, Finland, Spain, Germany and Ireland), Asia (China, Hong Kong, Thailand, Vietnam and Japan), and Oceania (Australia and New Zealand) (Staats *et al.*, 1997; Wertheim *et al.*, 2009). Although the most frequent clinical manifestation of the infection in swine is meningitis, other clinical features, such as arthritis, endocarditis, pneumonia, abortion and vaginitis, commonly occur (Gottschalk *et al.*, 2007). Without treatment, the mortality rate can reach 20% (Cloutier *et al.*, 2003). *S. suis* has additionally been isolated

from other species such as dogs, cats, horses, ruminants and humans (Devriese *et al.*, 1990; Devriese & Haesebrouck, 1992; Keymer *et al.*, 1983; Muckle *et al.*, 2010; Staats *et al.*, 1997).

The isolates from diseased pigs in many countries were often limited to some serotypes (Gottschalk *et al.*, 2007; Wisselink *et al.*, 2000). Besides serotype 2, serotypes 9, 1, 1/2 and 14 were also isolated from diseased pigs in European countries (Princivalli *et al.*, 2009; Wisselink *et al.*, 2000). The notable numbers of strains belonging to serotypes 14, 9, 7, 11, 8 and 1 were found in Brazil (Costa *et al.*, 2005) and serotypes 3, 4, 8, 5, 7, 1/2 were isolated from infected pigs in China (Wei *et al.*, 2009). However, the recent study (Gottschalk *et al.*, 2013) reported that the prevalence of serotype 2 in Canada was almost the same as that of serotype 3 and the prevalence of serotype 2 in North America was still relatively low compared to European and Asian countries, suggesting that the difference of serotype distribution may result from the geographical location. Recently, followed by serotype 2, serotype 7 has been found to be the most predominant serotype circulating in slaughter pigs in China (Wang *et al.*, 2013). Because the serotypes in human isolates correlate with those predominant in pigs in the same area, it is suggested that not only the potential cause of disease transmission in the herd, the healthy carrier pigs may also be the important source to spread *S. suis* to humans. Moreover, according to the analyses by multilocus sequence typing in a study by Onishi *et al.* (2012), *S. suis* isolates from pigs with endocarditis mostly belong to sequence type 1 and 28 complexes that are also the potential hazardous groups causing serious infection in humans. This result emphasized public health significance of *S. suis* lurking in porcine endocarditis.

Since the first human case was reported in Denmark in 1968, the numbers of human case of *S. suis* infection have increased in many countries, particularly the area where the pig density is high (Wertheim *et al.*, 2009). In the summer of 2005, the largest recorded outbreak

of *S. suis* serotype 2 infection which involved 215 cases and 39 deaths, occurred in Sichuan Province, China. Three clinical features which were sepsis, meningitis and streptococcal toxic shock syndrome (STSS) were observed in this outbreak (Yu *et al.*, 2006). All patients were backyard pig farmers who were exposed to *S. suis* via direct contact with blood or tissue of infected pigs during slaughtering process (Yu *et al.*, 2006). Sporadic human cases have also commonly occurred in many countries. The main *S. suis* infection route in human is by direct contact with carrier pigs, sick pigs or contaminated pork via injured skin or injured mucosa. Thus, *S. suis* infection in human is an occupational disease and the persons who work in close contact with pigs, such as pig farmers, abattoir and meat-processing workers, and veterinarians, have a high risk (Lun *et al.*, 2007). Moreover, *S. suis* was isolated from 6.1% of raw pork from 3 of the 6 wet markets in Hong Kong (Ip *et al.*, 2007) and human *S. suis* infections reported from Thailand and Vietnam were often caused by the consumption of raw or undercooked pork, pigs' blood or their products (Fongcom *et al.*, 2009; Nghia *et al.*, 2011; Takeuchi *et al.*, 2012). In addition to direct contact with pigs, the processing and consuming of uncooked pork or pork products are risk factors for the infection.

Meningitis is the most common clinical feature in human *S. suis* infection, whereas septicemia, endocarditis, arthritis, peritonitis, pneumonia, uveitis and endopthalmitis have also been reported (Segura, 2009). Mild to severe hearing loss is one of the distinct sequelae after recovery from meningitis and the prognosis for hearing is guarded (Wertheim *et al.*, 2009). Although the haemorrhagic labyrinthitis detected by magnetic resonance imaging was suggested to be the cause of deafness in *S. suis* meningitis patient (Tan *et al.*, 2010), the exact cause of deafness remains unknown (Choi *et al.*, 2012; Gottschalk *et al.*, 2007). Not only serotype 2 which is the most common cause in human infection, serotypes 1, 4, 5, 14, 16 and 24 have also been reported in the patients (Kerdsin *et al.*, 2011; Nghia *et al.*, 2008; Takeuchi

*et al.*, 2012; Wertheim *et al.*, 2009). Undoubtedly, *S. suis*, a major cause of human bacterial meningitis in Asian countries, is considered to be an important emerging zoonotic pathogen.

The virulence factors of *S. suis* have intensively been studied in the last few decades and many potential virulence factors have been identified including capsule, muramidase-released protein (MRP) (Smith *et al.*, 1992), extracellular protein factor (EF) (Vecht *et al.*, 1992), suilysin (Jacobs *et al.*, 1994), several adhesins (Baums & Valentin-Weigand, 2009), and hyaluronate lyase (Allen *et al.*, 2004). Although the precise role of MRP and EF is not clearly identified, these putative virulence factors are frequently associated with highly virulent serotype 2 strains and commonly served as virulence markers in some parts of the world (Fittipaldi *et al.*, 2012; Silva *et al.*, 2006).

Each putative virulence factor was proposed to play a role in each step of the pathogenesis of the infection. Due to the requirement of adhesion during colonization, adhesins such as fibronectin- and fibrinogen-binding protein (Fbps) (de Greeff *et al.*, 2002), enolases (Esgleas *et al.*, 2008) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Brassard *et al.*, 2001; Brassard *et al.*, 2004) are potentially involved in the virulence. After entering blood circulation, capsule which has been proved to protect the bacterial cells from host immune system is required for the bacterial survival and dissemination (Fittipaldi *et al.*, 2012). On the basis of the mutagenesis technique and the virulence attenuation of knockout mutants in the different experimental infection models, many factors or genes were additionally proposed to drive the full virulence of *S. suis* strains (Aranda *et al.*, 2010; Pan *et al.*, 2009; Vanier *et al.*, 2009; Wilson *et al.*, 2007; Zhang *et al.*, 2010). Moreover, a candidate pathogenicity island was proposed from the sequencing of the highly invasive *S. suis* serotype 2 strains from Chinese outbreak (Chen *et al.*, 2007). However, the putative virulence factors mostly exist in both virulent and avirulent strains and there may not be a universal virulence

factor for all *S. suis* strains (Fittipaldi *et al.*, 2012). Therefore, multiple virulence factors are essential for *S. suis* to achieve the infection.

Among the virulence determinants which have been described so far, capsular polysaccharide (CP) is thought to play an important role for effective systemic infection in various species of bacteria including *S. suis* and other streptococci (Corbett & Roberts, 2009). However, the unencapsulated and nontypeable streptococci have increasingly been isolated from clinical cases (Bonifait *et al.*, 2010; Crum *et al.*, 2004; Martin *et al.*, 2003; Melchiorre *et al.*, 2012; Sellin *et al.*, 1992). Katsumi et al. (1997) reported that 61.6% of *S. suis* isolates from slaughtered pigs with endocarditis were untypeable. Unlike isolates from other types of *S. suis* diseases, it was noticed that many *S. suis* isolates from pigs with endocarditis which possessed serotype 2 (or 1/2)-specific gene could not be agglutinated with either anti-serotype 1 or anti-serotype 2 sera, suggesting that these isolates may have frequently lost their ability to synthesize capsule. Although the unencapsulated and nontypeable *S. suis* isolates have previously been reported, the cause of unencapsulation and characteristics of these isolates remained largely unknown. Therefore, it is very interesting to elucidate the mechanism of this phenomenon.

This study aims to verify the loss of capsule, which occurs in many *S. suis* isolates from porcine endocarditis cases. In addition, for better understanding of the unencapsulation mechanism and its biological significance, the genetic backgrounds and potential benefit of the unencapsulation in the pathogenesis of infective endocarditis are investigated. The results from this study additionally demonstrate the regions in a capsular polysaccharide synthesis (*cps*) gene cluster in which there are the mutations related to the viability of *S. suis* and the potential benefit of the unencapsulation to facilitate the persistence of *S. suis* in host and the establishment of the infection.

# Chapter 1

# Loss of capsule in *S. suis* isolates from porcine endocarditis

## 1.1. Introduction

*Streptococcus suis* is a zoonotic pathogen that causes serious diseases, including meningitis, arthritis, septicemia and endocarditis, in swine and humans (Gottschalk *et al.*, 2007). *S. suis* strains are classified into different serotypes according to the antigenicity of their CPs (Higgins & Gottschalk, 2006). Among them, serotype 2 has been predominantly isolated from both infected pigs and patients in many countries (Wertheim *et al.*, 2009). Production of *S. suis* capsule is mediated by *cps* genes clustered in a single locus of the genome. Among the genes in the *cps* gene clusters identified to date (Holden *et al.*, 2009; Okura *et al.*, 2013; Smith *et al.*, 1999a; Smith *et al.*, 2000), *cps2J* existed only in strains of serotypes 2 and 1/2; 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.

Endocarditis caused by *S. suis* is often found in adult pigs, particularly in slaughter houses. In Japan, most of the endocarditis isolates are *cps2J*-positive by PCR (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 untypeable by agglutination tests (Katsumi *et al.*, 1997), whereas a high percentage of isolates from meningitis and pneumonia were serotypeable (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), these observations imply that many endocarditis isolates, especially those of serotypes 2 and 1/2, often lose their ability to synthesize capsules.

For confirmation of this speculation, in this chapter, the capsular production of *cps2J*-positive isolates from endocarditis and meningitis is investigated. Furthermore, the genetic backgrounds of 43 representative unencapsulated isolates are examined.

## 1.2. Materials and methods

### 1.2.1. Bacterial isolates, 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 isolated from heart valve vegetations of pigs with endocarditis in regional Livestock Hygiene Service Centers in Japan between 1994 and 2009. Thirty-two isolates, including 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 Livestock Hygiene Service Centers or National Institute of Animal Health in Ibaraki, Japan between 1989 and 2006. All isolates were stored in Luria-Bertani (Difco Laboratories, Becton Dickinson, Sparks, Maryland, U.S.A.) broth containing 30% glycerol at -80 ºC and minimally passaged for the experiments to avoid changing the key traits including capsule production. In addition, *S. suis* strains 204 (serotype 1 field isolate) (Sekizaki *et al.*, 2001) and S735 (NCTC $10234^T$; serotype 2 reference strain) were used for the production of rabbit antiserum. *Enterococcus faecalis* NCTC 775 was used as a control for PCR analysis. *S. suis* and *E. faecalis* were cultured in Todd Hewitt broth (THB; Difco Laboratories) or agar (THA) at 37 ºC in air plus 5 % $CO_2$ for 16 h. *Escherichia coli* strain was cultured in Luria-Bertani (Difco Laboratories) broth or agar at 37ºC. When required, spectinomycin was added to the medium for *E. coli* at 50 μg/ml and for *S. suis* at 100 μg/ml.

### 1.2.2. Confirmation of *S. suis* and *cps2J*-positive *S. suis* isolates

Identification of *S. suis* field isolates was confirmed by species-specific PCR for *S. suis* that targeted the glutamate dehydrogenase gene (*gdh*) (Okwumabua *et al.*, 2003) or sequencing of the 16S rRNA gene. The presence of *cps2J* was determined by PCR as described previously (Silva *et al.*, 2006). Template DNA was prepared by InstaGene Matrix (Bio-Rad Laboratories, Hercules, California, U.S.A.), and PCR reactions were performed

using *Ex Taq* polymerase (Takara Bio, Otsu, Shiga, Japan) following the manufacturers' recommendations. Primers used for *gdh* and *cps2J* amplifications and expected size of PCR products are shown in Table 1.1. The following PCR cycling was used; started at 94° C for 2 min, repeating 30 cycles of 94° C for 20 sec, 55° C (for *gdh*) or 58° C (for *cps2J*) for 10 sec, 72° C for 30 sec and final extension at 72 ºC for 1 min. The PCR products were visualized by electrophoresis on 1% agarose gel following standard procedures.

### 1.2.3. Production of rabbit antisera

Rabbit anti-serotype 1 and anti-serotype 2 polyclonal sera were prepared by immunizing rabbits with formalin-killed *S. suis* strains 204 and S735, respectively, according to the procedure described previously (Higgins & Gottschalk, 1990). Briefly, rabbits weighing 3 kg were given 3 injections per week of increasing numbers of bacteria during 4 weeks as follows: first week, 2-4 x $10^9$ CFU; second to fourth week, 4-8 x $10^9$ CFU. 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.

### 1.2.4. Serotyping

The capsular antigens of all *cps2J*-positive isolates cultured on THA plates were extracted by autoclaving the cells in Dulbecco's phosphate-buffered saline (DPBS) at 121 ºC for 15 min and tested with anti-serotypes 1 and 2 sera. The coagglutination technique was applied as previously described (Gottschalk *et al.*, 1989; Han *et al.*, 2001). The capsular antigen and coagglutination reagent were mixed on black paper slide and the reaction was judged as positive when agglutination occurred within 5 min. As for isolates with a negative-

agglutination reaction, further coagglutination tests were performed to verify the absence of capsule using isolates subcultured under the condition recommended to enhance capsular production (Gottschalk *et al.*, 1993).

### 1.2.5. 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 the THA plate, washed with phosphate-buffered saline (PBS, 0.01 M, pH 7.2), and incubated with anti-serotype 2 serum at 4 ºC 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, postfixed with 1 % osmium tetroxide at 4 ºC 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, Tokyo, Japan). Ultrathin sections were stained with uranyl acetate and lead citrate prior to examination with a transmission electron microscope (H-7500; Hitachi, Tokyo, Japan).

### 1.2.6. PCR analysis of genes in the *cps* gene clusters and sequencing of mutated regions

Twenty-one primer sets covering the almost entire *cps* gene cluster of serotype 2 (*cps2A-2S*) (Table 1.2 and Figure 1.1) 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 PCR reactions were performed using *Ex Taq* polymerase (Takara Bio) according to the manufacturer's instructions. The conditions of the PCR analysis consisted of pre-denaturing at 95 ºC for 5 min, 30 cycles of 20 sec at 95 ºC, 10 sec at 55 ºC, 1 min at 72 ºC, and final extension at 72 ºC

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 analyzed by electrophoresis on 1 % agarose gel and/or a MultiNA microchip electrophoresis system (Shimadzu Corporation, Kyoto, Japan).

The PCR products of sizes different from those of *S. suis* P1/7 were purified by a QIAquick PCR purification kit (Qiagen, Hilden, Germany) 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. For the isolates which gave expected PCR amplifications, *LA Taq* and *Ex Taq* polymerase (Takara Bio) were used for amplification of genes in their *cps* gene clusters. The PCR products were sequenced by a BigDye terminator v3.1 cycle sequencing kit using a 3130xl Genetic Analyzer (Applied Biosystems, Foster City, California, U.S.A.). Sequencher Ver. 4.8 (Hitachi Software Engineering, Yokohama, Japan) 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 CLUSTALW 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.

### 1.2.7. Bacterial strains, plasmid, primers and DNA techniques for the construction of *cps* gene expression vectors and complementation analysis

Plasmids and bacterial strains used for the construction of expression vectors are listed in Table 1.3. Genomic DNA of *S. suis* was extracted as described previously (Mogollon *et al.*, 1990), and plasmid DNA of *E. coli* was purified using the Illustra™ plasmid Prep Mini Spin Kit (GE Healthcare, Freiburg, Germany) according to the manufacturer's

recommendations. Restriction enzymes and DNA-modifying enzymes were purchased from Takara Bio and used according to the manufacturer's recommendations. The transformation of *E. coli* was carried out following the standard procedures (Sambrook & Russell, 2001) and *S. suis* was transformed by electroporation as described previously (Takamatsu *et al.*, 2001b).

For construction of *cps2E*, *cps2F*, and *cps2K* expression vectors, designated pCps2E, pCps2F, and pCps2K, respectively, the entire *cps2E*, *cps2F*, and *cps2K* genes were amplified from genomic DNA of *S. suis* strains P1/7 and/or 89/1591 by PCR with primers listed in Table 1.4 and iProof HF Master mix (Bio-Rad Laboratories), digested by BamHI and EcoRI (for pCps2E and pCps2F) or PstI and EcoRI (for pCps2K), and cloned into the respective sites of pMX1. For construction of the *cps2F-cps2K* expression vector, designated pCps2F-2K, *cps2F* and *cps2K* were individually amplified by PCR with the primers listed in Table 1.4, fused by overlap-extension PCR (Warrens *et al.*, 1997), and subsequently cloned into pMX1 via PstI and EcoRI sites. After introducing *E. coli* strain MC1061, the direction and sequences of the cloned genes were verified by PCR and sequencing. The resultant expression vectors were then introduced into unencapsulated *S. suis* isolates, and restoration of the capsular expression was examined by the coagglutination test and TEM.

## 1.3. Results

### 1.3.1. Loss of capsule in *cps2J*-positive endocarditis isolates

A total of 288 isolates which were confirmed to be *cps2J*-positive *S. suis* were examined for capsular production by the coagglutination test. All 32 isolates from meningitis exhibited agglutination with anti-serotype 2 serum, and among them, four were also agglutinated with anti-serotype 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 anti-serotype 2 serum, 86 (34 %) isolates were not agglutinated with either

anti-serotype 1 or anti-serotype 2 sera, suggesting that these isolates have lost their capsule. The two coagglutination-positive (P1/7 and NL333) and four coagglutination-negative (NL146, NL194, NL240 and NL290) strain and isolates were selected and examined visually by TEM to confirm the coagglutination results. As shown in Figure 1.2, cells of both coagglutination-positive strain and isolate 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.

### 1.3.2. Structural alterations occurred in the *cps* gene clusters of the unencapsulated isolates

For investigation of the genetic backgrounds affecting capsular expression, 43 representative unencapsulated isolates were randomly selected and analyzed for their genes in *cps* gene clusters by PCR analysis. As shown in Table 1.5, 18 isolates gave no or unexpected amplification with at least one of the primer sets (Figure 1.3), indicating structural alterations in the *cps* gene clusters. Nucleotide sequencing of those regions showed that genes in the *cps* gene clusters of 8 and 10 isolates had deletions and insertions, respectively (Figure 1.4). However, no apparent alteration was found in the *cps* regions of the other 25 isolates by PCR analysis.

Three isolates (NL157, NL191 and NL280) had a partial deletion in the *cps2P*, *cps2E*, and *cps2G* genes, respectively, whereas five (NL204, NL217, NL268, NL290, and NL319) had a deletion of multiple genes, as shown in Figure 1.4. Among the five isolates above, a large deletion (more than 17.5 kb fragment including the *cps2P*, *cps2Q, cps2R* and *cps2S* genes) was found in NL217. In addition, this isolate had a 192-bp deletion in the *cps2F* gene. In NL204 and NL290, more than 3-kb deletions were found in almost the same region

(*cps2A*, *cps2B*, *cps2C*, *cps2D* and *cps2E* genes), while SSU0514-*cps2A* and *cps2F*-*G* regions were lost in NL268 and NL319, respectively.

IS or putative IS elements also disrupted *cps* genes (Figure 1.4 and Table 1.6). The IS elements found in NL85 and NL194 were classified into the ISL*3* family, while those of NL155, NL171, NL198, NL201 and NL255 were classified into the IS*110* family. Although a putative IS element found in NL184 did not show significant similarity to any other known IS elements by means of blastn search, the closest relative was proposed to be ISTel*1* of *Thermosynechococcus elongatus* BP-1, which belongs to the IS*481* family (Nakamura *et al.*, 2002) by the IS finder search. However, the sequences inserted in the *cps* gene clusters of NL176 and NL179 encoded putative reverse transcriptases that constitute group II introns (Michel & Ferat, 1995).

Notably, most of the deletion and insertion were found in the *cps2A*-*2G* and *cps2P*-*cps2S* regions, particularly in *cps2E*, as 7 of 10 insertions were found in this gene. On the other hand, the *cps2H*- *cps2O* region of the isolates analyzed was rarely affected.

### 1.3.3. Capsule loss in *S. suis* isolates due to mutations in *cps2EF*

The entire *cps2* gene clusters (*cps2A*-*2S*) of three representative isolates (NL119, NL257, and NL278) were sequenced to reveal the potential cause of unencapsulation of the 25 endocarditis isolates that gave expected PCR amplifications. Compared with the sequence of *S. suis* strain P1/7, NL257 had a 4-bp insertion resulting in frameshift mutation in *cps2E*, and the other two had single nucleotide substitutions in *cps2F*, leading to single amino acid substitutions of the gene products (Figure 1.5 and Table 1.7), whereas the other genes in the clusters were intact. For investigation whether these mutations impeded capsular production, NL257 was transformed with pCps2E and NL119 and NL278 were transformed with pCps2F. In all three transformants, the coagglutination test using anti-serotype 2 serum

showed positive reactions, and TEM analysis confirmed the distinct layer of capsule on the cell surfaces, whereas capsular material was not obviously seen on the surface of cells from the parent strains (Figure 1.6), which affirmed that mutations in the *cps2EF* region caused capsule loss in the three isolates.

Because these results together with the results described above (1.3.2) implied that mutations affecting capsular production may often occur in the *cps2EF* region, genes of the *cps2EF* region of the remaining 22 unencapsulated isolates were sequenced and compared with those of *S. suis* strains P1/7 and 89/1591 (accession no.: AAFA03000010). As shown in Figure 1.5, six and four of the 22 isolates had frame shift mutations in *cps2E* and *cps2F*, respectively, while single nucleotide substitutions within *cps2E* and *cps2F* were found in two and five isolates, respectively. Among the seven nucleotide substitutions, three created termination codons (nonsense mutations), and the other four generated amino acid substitutions (missense mutations). The *cps2EF* sequencing results additionally showed that one isolate carried 81-bp deletion in *cps2F* and only four isolates had intact *cps2E* and *cps2F*.

The complementation analyses by introducing pCps2E or pCps2F to the isolates and examining the capsular expression by coagglutination test were subsequently performed to verify that the mutations found in *cps2EF* region were responsible for the unencapsulation. Because nonsense mutations found in three isolates, frameshift mutations found in seven isolates, and an 81-bp deletion found in one isolate resulted in truncation or significant shortening of the products, these mutations were supposed to be involved in their loss of capsule; therefore, only two isolates (NL100 and NL240) were investigated. On the other hand, because frameshift mutations due to the deletion of four bases (TAAG) at the site of the termination codon of *cps2E* found in three isolates (NL208, NL219 and NL342) resulted in the extension of Cps2E, these three were included for the analysis to verify influence of the

putative structural conformation change of Cps2E. All missense mutations were also investigated.

As shown in Figure 1.5, except for NL208, from which no transformant was obtained, all isolates with nonsense or frameshift mutations showed positive coagglutination reactions after the introduction of pCps2E or pCps2F, which indicated that these nonsense and frameshift mutations in the *cps2EF* region caused capsule loss of the isolates. Among four isolates carrying missense mutations, transformants from two isolates (NL122 and NL295) showed positive coagglutination reactions, indicating that at least two of the four missense mutations affected function of the genes. However, because transformants from the other two isolates with missense mutations (NL132 and NL143) gave negative coagglutination results, the cause of unencapsulation in these two as well as four isolates (NL174, NL175 NL322, and NL345), which carried intact *cps2E* and *cps2F*, was unclear. Therefore, the almost entire *cps* gene clusters of the six isolates were further sequenced. As shown in Figure 1.7, NL174 and NL175 had the same single frameshift mutation in *cps2H*, while NL322 and NL345 had 50-bp and 23-bp deletions in *cps2G* and *cps2H*, respectively, suggesting that these mutations caused loss of their capsule. In addition to the mutations in the *cps2EF* region, NL132 had another frameshift mutation in *cps2H*, while NL143 harbored a 9-bp insertion in *cps2K*, leading to the duplication of three amino acid residues (Asn His Trp) within Cps2K. Because the capsule of NL143 was restored only after introduction of both intact *cps2F* and *cps2K*, the capsule loss in NL143 was indicated to result from mutations in both *cps2F* and *cps2K*.

These results together with the results described in 1.3.2 indicated that at least 32 (74.4%) of 43 representative unencapsulated isolates have lost their capsules because of the mutations in *cps2EF* region (Table 1.8). These facts confirmed that mutations in the *cps2EF* region caused unencapsulation in most of the *S. suis* isolates from porcine endocarditis.

## 1.4. Discussion

In addition to the molecular basis for serotyping in *S. suis* (Higgins & Gottschalk, 2006), 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). Moreover, capsule loss reduced the virulence of *S. suis* in both mice and swine models of infection (Smith *et al.*, 1999a). However, in this chapter, approximately one third of the endocarditis isolates from swine were demonstrated to lose the ability to produce capsules. Therefore, it is suggested that the loss of capsule in these isolates may provide some advantageous properties to the pathogenesis of infective endocarditis in swine.

Because encapsulated isolates were retrieved from 66 % of the porcine endocarditis cases analyzed 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 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, the 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* (Figure 1.4 and Table 1.6) were found in this study. 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 may suggest the transmission of unencapsulated *S. suis* strains among pigs and farms, although the possibility that the inserted position was the preferential site for the IS elements and insertion events occurred

independently in each isolate cannot be completely ruled out. Notably, most of the deletion and insertion found in the unencapsulated isolates occurred in the *cps2A-2G* 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 analysis in the 25 unencapsulated isolates. As previously reported by Willenborg *et al.* (2011), the 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, 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 (unpublished observations), suggesting that *ccpA* of the unencapsulated isolates was intact. Therefore, their *cps* gene clusters may be affected by point mutations that could not be detected by PCR analysis. As expected, the sequencing and complementation results of the remaining 25 isolates showed that most of them had nucleotide alterations of a single or a few base pairs in the *cps2EF* region, causing frameshift, missense or nonsense mutation(s) of the genes.

Alternatively, it is conceivable that their capsular expression was negatively regulated by unknown mechanisms. In fact, phase variation of the capsular expression has previously been reported in a group B *Streptococcus* (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 were found in other Gram-positive and -negative bacteria (van der Woude & Baumler, 2004). Although such variations have not been reported in *S. suis*, capsular expression may be reversible in some unencapsulated *S. suis* isolates. Moreover, even in unencapsulated isolates, which had mutations affecting capsular expression, capsule production could be restored by acquiring functional genes via horizontal gene transfer,

additional point mutations, and 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* gene clusters of NL176 and NL179 were similarly disrupted by the insertion of putative group II intron within *cps2M*. Because *cps2M* is considered to be a pseudogene, it cannot be assumed that the disruption of *cps2M* itself caused the loss of capsular production in these isolates. Thus, in the two isolates, additional point mutations and/or negative regulation of capsular expression may have occurred in addition to the insertion events. Alternatively, the expression of downstream genes may have been affected by a polar effect caused by insertion of the group II intron.

Although the capsule is thought to be an important virulence factor for *S. suis*, the results in this chapter demonstrated that about 34% of the endocarditis isolates from swine lost the ability to produce capsule. According to the results from PCR analysis, sequencing and complementation, at least 32 of 43 representative unencapsulated isolates have lost their capsules because of the mutations involving *cps2EF* region.

## 1.5. Summary

In this chapter, 288 *cps2J*-positive *S. suis* isolates from diseased pigs (256 from endocarditis and 32 from the brains or meningitis) were analyzed. All isolates from meningitis were shown to be encapsulated by coagglutination tests and TEM, whereas 86 isolates (34%) from endocarditis were identified to be unencapsulated. Among 43 unencapsulated endocarditis isolates that were randomly selected for PCR analysis, 10 and 8 isolates were demonstrated to have apparent insertions and deletions, respectively, in their *cps* gene clusters. The *cps* gene clusters of the rest of 25 isolates were analyzed by sequencing and complementation. The results demonstrated that at least 32 of 43 representative unencapsulated isolates have lost their capsules because of the mutations in *cps2EF* region. Intriguingly, the mutation within the *cps2IJ* and *cps2NO* regions was rarely found in the isolates analyzed in this study. Since capsule is considered as a virulence determinant in *S. suis*, the unencapsulation due to these structural alterations may provide some benefits to cause endocarditis in swine.

**Table 1.1.** Primers used for *gdh* and *cps2J* amplifications and expected size of PCR products.

| Target gene | Primer | Sequence | Expected size of PCR product |
|---|---|---|---|
| *gdh* | Forward | 5'-GCAGCGTATTCTGTCAAACG-3' | 688 bp |
| | Reverse | 5'-CCATGGACAGATAAAGATGG-3' | |
| *cps2J* | Forward | 5'-TTTGTCGGGAGGGTTACTTG-3' | 498 bp |
| | Reverse | 5'-TTTGGAAGCGATTCATCTCC-3' | |

**Table 1.2.** Primer sets for PCR analysis.

| Primer pairs | Forward | Sequence (5'-3') | Reverse | Sequence (5'-3') | Product size in strain P1/7 |
|---|---|---|---|---|---|
| 1 | cps2-F1 | AGGTAAAAGTCTAGGAAGGT | cps2-R1 | ACGGACTGAATCGCTGCCTT | 1455 bp |
| 2 | cps2-F2 | TTAATCGACTTGGTGGGTGG | cps2-R2 | GAAGAAACCAGGCATGACTG | 1451 bp |
| 3 | cps2-F3 | AGCGGAAGAACCAACCACTC | cps2-R3 | CGCCAATTAAAGCAGGCTTC | 1441 bp |
| 4 | cps2-F4 | CGAAGCTTATCGTCAAGGTG | cps2-R4 | TCATCAAGATGTGACTAGGC | 1472 bp |
| 5 | cps2-F5 | CGTGGTGCCGTGTATTTCAC | cps2-R5 | CATGACCGTCTGGGTTTACG | 1520 bp |
| 6 | cps2-F6 | GGACCATCTGGTCAGACATT | cps2-R6 | CCTACGTAGAATTGGATAGT | 1476 bp |
| 7 | cps2-F7 | GGAACGAACCCATCTTTACT | cps2-R7 | ACTTTGAGGGAGGTGTAGAC | 1412 bp |
| 8 | cps2-F8 | GAGTGGCGAGTAGTAGAATT | cps2-R8 | CGATATTGTTCTCCATAGTAGC | 1490 bp |
| 9 | cps2-F9 | GAGGCATATAATCAGTATCG | cps2-R9 | TTGCTGACATATTCCGATAG | 1492 bp |
| 10 | cps2-F10 | GGTACAGGTGTAGACTTGTC | cps2-R10 | GTTGATTCCATAGTAGATATAG | 1422 bp |
| 11 | cps2-F11 | TGATTCTTACGCTCATCGCG | cps2-R11 | AGAGGACGTTCGTTTAATAC | 1480 bp |
| 12 | cps2-F12 | AAGAGGTGCGAGACTTAGGA | cps2-R12 | AAGCTTCTTTTGCTGTTTGC | 1398 bp |
| 13 | cps2-F13 | TCAGGCTGTTCTGAGCGGCA | cps2-R13 | ATTGCTGGAATCTTCTGTCC | 1449 bp |
| 14 | cps2-F14 | ATCGGGTCCAGGGAGTTGGG | cps2-R14 | GGATCTGATACATCGTATGG | 1464 bp |
| 15 | cps2-F15 | TCAGAAATGTATAAGGGGGG | cps2-R15 | GTTCCAATCGTATAGACGAG | 1481 bp |
| 16 | cps2-F16 | AGCAAGGGCGATAGTAGCGG | cps2-R16 | CAGATAGGAAGCAGTCGTTG | 1566 bp |
| 17 | cps2-F17 | TGGGCTTCATTTTCGAAAGG | cps2-R17 | GGTATTCTGCCTTTGGTGCG | 1502 bp |
| 18 | cps2-F18 | CTCTACCATGAAAATTGTGC | cps2-R18 | ATCCTACGCTTGTCCGCTTC | 1552 bp |
| 19 | cps2-F19 | CTGGACAATGAAATGGAAGG | cps2-R19 | CATGGTTGAGGCCTGTACAG | 1466 bp |
| 20 | cps2-F20 | GGAGGATAACACAGCCGAAG | cps2-R20 | CTAACAACGTGGTCAGCTTG | 1533 bp |
| 21 | cps2-F21 | CAGTGATTCAATGTATCGAG | cps2-R21 | CCAACATAGTCTAGCCACTG | 1400 bp |

**Table 1.3.** Bacterial strains and plasmids used in this study.

| Strain or plasmid | Relevant characteristics | Source or reference |
|---|---|---|
| *S. suis* strains | | |
| P1/7 | Wild-type strain isolated from a pig with meningitis | (Slater *et al.*, 2003) |
| 89/1591 | Wild-type strain isolated from a pig with septicemia and meningitis | (Salasia *et al.*, 1995) |
| *E. coli* strain | | |
| MC1061 | Host for pMX1 derivatives | (Casadaban & Cohen, 1980) |
| Plasmids | | |
| pMX1 | $Spc^r$, pSSU1 *ori*, the *malX* promoter of *S. suis*, the derivative of pSET2 | (Okura *et al.*, 2011) |
| pCps2E | pMX1 carrying the intact *cps2E* gene | This study |
| pCps2F | pMX1 carrying the intact *cps2F* gene | This study |
| pCps2K | pMX1 carrying the intact *cps2K* gene | This study |
| pCps2F-2K | pMX1 carrying intact *cps2F* and *cps2K* genes | This study |

**Table 1.4.** Primers used for construction of *cps* gene expression vectors.

| Primer | Sequence (5'-3') | Description | PCR program[a] |
|---|---|---|---|
| For construction of pCps2E | | | |
| pCps2E-F | AAACGGATCCCAAGTACAGTAACCTCATAG | BamHI site is underlined. | 98°C 2min - 98°C 20sec, 60°C 10sec, 72°C 1min (30 cycles) - 72°C 2min |
| pCps2E-R | CACCGAATTCCCATACTTTGCTGGTATTCC | EcoRI site is underlined. | |
| For construction of pCps2F | | | |
| pCps2F-F | TTGTGGATCCAGGGAAGTAAGTAAGACTCC | BamHI site is underlined. | 98°C 2min - 98°C 20sec, 60°C 10sec, 72°C 1min (30 cycles) - 72°C 2min |
| pCps2F-R | TGCCCCATAGAATTCTGCTCCAGCATGGAG | EcoRI site is underlined. | |
| For construction of pCps2K | | | |
| pCps2K-F | TTACCTGCAGTATTAAACGAACGTCCTCTG | PstI site is underlined. | 98°C 2min - 98°C 20sec, 60°C 10sec, 72°C 1min (30 cycles) - 72°C 2min |
| pCps2K-R | TACTGAATTCTTACCGAGTACTACTTTCAC | EcoRI site is underlined. | |
| For construction of pCps2F-2K | | | |
| 1-pCps2FK | GGAGATATGAGTCTAGTCGG | For amplification of *cps2F* and its flanking regions | 98°C 2min - 98°C 10sec, 55°C 10sec, 72°C 1min (30 cycles) - 72°C 2min |
| 2-pCps2FK | CCTACGTAGAATTGGATAGT | For amplification of *cps2F* and its flanking regions | |
| 3-pCps2FK | AAGAGGTGCGAGACTTAGGA | For amplification of *cps2K* and its flanking regions | 98°C 2min - 98°C 10sec, 55°C 10sec, 72°C 1min (30 cycles) - 72°C 2min |
| 4-pCps2FK | ATTGCTGGAATCTTCTGTCC | For amplification of *cps2K* and its flanking regions | |
| 5-pCps2FK | ATCTCTGCAGGCTGGACCTAACATACATTG | Used for amplification of *cps2F* and generation of fused fragment to vector. PstI site is underlined. | 98°C 2min - 98°C 10sec, 60°C 10sec, 72°C 1min (30 cycles) - 72°C 2min |
| 6-pCps2FK | GCGATTCATCTCCATAATGCTGGCACTAGG | Used for amplification of *cps2F* and generation of fused fragment to *cps2K*. | |
| 7-pCps2FK | CCTAGTGCCAGCATTATGGAGATGAATCGC | Used for amplification of *cps2K* and generation of fused fragment to *cps2F*. | 98°C 2min - 98°C 10sec, 60°C 10sec, 72°C 1min (30 cycles) - 72°C 2min |
| 8-pCps2FK | TACTGAATTCTTACCGAGTACTACTTTCAC | Used for amplification of *cps2K* and generation of fused fragment to vector. EcoRI site is underlined. | |

[a] All PCRs were performed by using iProof HF Master mix (Bio-Rad Laboratories).

**Table 1.5.** PCR analysis results.

| Strain/ Isolate | Primer pairs | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 |
| *S. suis* P1/7 | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| NL85 | L | L | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| NL155 | + | + | + | L | L | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| NL157 | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | S | - | + | + |
| NL171 | + | + | + | L | L | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| NL176 | + | + | + | + | + | + | + | + | + | + | + | + | + | - | + | + | + | + | + | + | + |
| NL179 | + | + | + | + | + | + | + | + | + | + | + | + | + | - | + | + | + | + | + | + | + |
| NL184 | + | + | + | L | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| NL191 | + | + | + | + | S | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| NL194 | + | + | + | L | L | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| NL198 | + | + | + | L | L | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| NL201 | + | + | + | L | L | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| NL204 | - | - | - | - | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| NL217 | + | + | + | + | S | S | + | + | + | + | + | + | + | + | + | + | + | - | - | - | - |
| NL255 | + | + | + | L | L | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| NL268 | - | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| NL280 | + | + | + | + | + | - | S | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| NL290 | - | - | - | - | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| NL319 | + | + | + | + | + | - | - | - | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Other isolates[a] | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| *E. faecalis* NCTC775 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |

+, PCR positive; -, PCR negative; L, amplified PCR products were larger than expected; S, amplified PCR products were smaller than expected.

[a] Other isolates include *S. suis* NL100, NL119, NL122, NL126, NL132, NL143, NL146, NL174, NL175, NL208, NL219, NL225, NL230, NL240, NL245, NL249, NL257, NL266, NL278, NL295, NL303, NL322, NL328, NL342 and NL345.

**Table 1.6.** Inserted sequences in the *cps* gene clusters of unencapsulated isolates.

| Isolate | Size of the inserted sequences (bp) | Accession no. of the inserted sequences | NCBI blastn results[a] | | IS Finder blastp results[b] | | DR[c] |
|---|---|---|---|---|---|---|---|
| | | | Nucleotide identity (%) | Description and function of close relative [Accession no.] | Amino acid identity (%) | Description and function of close relative [Accession no.] | |
| NL85[d] | 1,415 | AB627103 | 1412/1415 (99%) | Transposase, IS*204*/IS*1001*/IS*1096*/IS*1165* family protein of *Streptococcus suis* strain ST3 [CP002633] | 347/417 (83%) | Putative transposase of ISSth*1* of *Streptococcus thermophilus* (ISL*3* family) [AY376237] | 8 |
| NL155[e] | 1,619 | AB627104 | 1617/1619 (99%) | Transposase of *Streptococcus suis* strain ST3 [CP002633] | 181/378 (47%) | Transposase of ISL*4* of *Lactobacillus delbrueckii* subsp. *bulgaricus* (IS*110* family) [AY040213] | 4 |
| NL171[e] | 1,619 | AB627105 | 1617/1619 (99%) | Transposase of *Streptococcus suis* strain ST3 [CP002633] | 181/378 (47%) | Transposase of ISL*4* of *Lactobacillus delbrueckii* subsp. *bulgaricus* (IS*110* family) [AY040213] | 4 |
| NL176[f] | 2,311 | AB627106 | 2297/2311 (99%) | Putative reverse transcriptase gene of *Streptococcus agalactiae* strain DSM2134 [EF990366] | 564/568 (99%) | RNA-direct DNA polymerase of *Streptococcus agalactiae* strain ATCC 13813 [EFV96300][g] | 0 |
| NL179[f] | 2,311 | AB627107 | 2297/2311 (99%) | Putative reverse transcriptase gene of *Streptococcus agalactiae* strain DSM2134 [EF990366] | 564/568 (99%) | RNA-direct DNA polymerase of *Streptococcus agalactiae* strain ATCC 13813 [EFV96300][g] | 0 |
| NL184 | 1,115 | AB627108 | NS[h] | NS[h] | 90/306 (29%) | Putative transposase of ISTel*1* of *Thermosynechococcus elongatus* strain BP-1 [NC_004113] | 25 |
| NL194[d] | 1,416 | AB627109 | 1376/1416 (97%) | Transposase, IS*204*/IS*1001*/IS*1096*/IS*1165* family protein of *Streptococcus suis* strain ST3 [CP002633] | 363/433 (83%) | Putative transposase of ISSth*1* of *Streptococcus thermophilus* (ISL*3* family) [AY376237] | 8 |
| NL198[e] | 1,619 | AB627110 | 1619/1619 (100%) | Transposase of *Streptococcus suis* strain ST3 [CP002633] | 181/378 (47%) | Transposase of ISL*4* of *Lactobacillus delbrueckii* subsp. *bulgaricus* (IS*110* family) [AY040213] | 4 |
| NL201[e] | 1,619 | AB627111 | 1617/1619 (99%) | Transposase of *Streptococcus suis* strain ST3 [CP002633] | 181/378 (47%) | Transposase of ISL*4* of *Lactobacillus delbrueckii* subsp. *bulgaricus* (IS*110* family) [AY040213] | 4 |
| NL255[e] | 1,619 | AB627112 | 1617/1619 (99%) | Transposase of *Streptococcus suis* strain ST3 [CP002633] | 181/378 (47%) | Transposase of ISL*4* of *Lactobacillus delbrueckii* subsp. *bulgaricus* (IS*110* family) [AY040213] | 4 |

[a] The blastn analysis was performed on the NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi) using the inserted sequences.

[b] The blastp analysis was performed on the IS finder website (http://www-is.biotoul.fr/) using the deduced amino acid sequences encoded in the inserted sequences.

[c] DR indicates the number of target base pairs duplicated on insertion.

[d] The inserted sequences were 97.3% identical but located at different positions.

[e] The inserted sequences were 99.8-100% identical and located at the same position.

[f] The inserted sequences were 100% identical and located at the same position.

[g] NCBI blastp result.

[h] NS, no significant similarity.

**Table 1.7.** Mutations found in 43 unencapsulated isolates analyzed in this study.

| No. | Isolate | Affected gene(s) | Types of mutation or structural alteration | Affected nucleotide(s) (Affected amino acid) |
|---|---|---|---|---|
| 1 | NL85 | *cps2A* | Insertion | IS element: 1415 bp |
| 2 | NL100 | *cps2F* | Nonsense[b] | T696G (Tyr232TERM) |
| 3 | NL119[a] | *cps2F* | Missense[b] | T490C (Cys164Arg) |
| 4 | NL122 | *cps2F* | Missense[b] | G52A (Gly18Ser) |
| 5 | NL126 | *cps2F* | Frame shift caused by insertion | TCCG |
| 6 | NL132[a] | *cps2E* | Missense | G1199A (Arg400Lys) |
| | | *cps2H* | Frame shift caused by deletion | TA |
| 7 | NL143[a] | *cps2F* | Missense[b] | G493T (Asp165Tyr) |
| | | *cps2K* | Insertion[b] | AATCATTGG (Asn His Trp) |
| | | *cps2R* | Missense | G496A (Gly166Arg) |
| 8 | NL146 | *cps2F* | Nonsense | T482A (Leu162TERM) |
| 9 | NL155 | *cps2E* | Insertion | IS element: 1619 bp |
| 10 | NL157 | *cps2P* | Deletion | 333 bp |
| 11 | NL171 | *cps2E* | Insertion | IS element: 1619 bp |
| 12 | NL174[a] | *cps2H* | Frame shift caused by deletion | A |
| 13 | NL175[a] | *cps2H* | Frame shift caused by deletion | A |
| 14 | NL176 | *cps2M* | Insertion | Putative group II intron: 2311 bp |
| 15 | NL179 | *cps2M* | Insertion | Putative group II intron: 2311 bp |
| 16 | NL184 | *cps2E* | Insertion | IS element: 1115 bp |
| 17 | NL191 | *cps2E* | Deletion | 68 bp |
| 18 | NL194 | *cps2E* | Insertion | IS element: 1416 bp |
| 19 | NL198 | *cps2E* | Insertion | IS element: 1619 bp |
| 20 | NL201 | *cps2E* | Insertion | IS element: 1619 bp |
| 21 | NL204 | *cps2ABCDE* | Deletion | 3215 bp |
| 22 | NL208 | *cps2E* | Frame shift caused by deletion | TAAG |
| 23 | NL217 | *cps2F* and *cps2PQRS* | Deletion | 192 bp and 17562 bp |
| 24 | NL219 | *cps2E* | Frame shift caused by deletion[b] | TAAG |
| 25 | NL225 | *cps2F* | Frame shift caused by insertion | CCAAA |
| 26 | NL230 | *cps2F* | Frame shift caused by insertion | A |
| 27 | NL240 | *cps2E* | Nonsense[b] | C1189T (Gln397TERM) |
| 28 | NL245 | *cps2E* | Frame shift caused by insertion | T |
| 29 | NL249 | *cps2E* | Frame shift caused by insertion | AGCA |
| 30 | NL255 | *cps2E* | Insertion | IS element: 1619 bp |

**Table 1.7. (continued)** Mutations found in 43 unencapsulated isolates analyzed in this study.

| No. | Isolate | Affected gene(s) | Types of mutation or structural alteration | Affected nucleotide(s) (Affected amino acid) | |
|---|---|---|---|---|---|
| 31 | NL257[a] | *cps2E* | Frame shift caused by insertion[b] | ATCT | |
| 32 | NL266 | *cps2E* | Frame shift caused by deletion | A | |
| 33 | NL268 | *cps2A* | Deletion | 1953 bp | |
| 34 | NL278[a] | *cps2F* | Missense[b] | T259C | (Ser87Pro) |
| 35 | NL280 | *cps2G* | Deletion | 410 bp | |
| 36 | NL290 | *cps2ABCDE* | Deletion | 3538 bp | |
| 37 | NL295 | *cps2F* | Missense[b] | T492G | (Cys164Trp) |
| 38 | NL303 | *cps2F* | Deletion | 81 bp | |
| 39 | NL319 | *cps2FG* | Deletion | 1719 bp | |
| 40 | NL322[a] | *cps2B* | Missense | G469A | (Asp157Asn) |
| | | *cps2G* | Deletion | 50 bp | |
| 41 | NL328 | *cps2F* | Frame shift caused by deletion | AG | |
| 42 | NL342 | *cps2E* | Frame shift caused by deletion[b] | TAAG | |
| 43 | NL345[a] | *cps2H* | Deletion | 23 bp | |
| | | *cps2N* | Missense | C706T | (Pro236Ser) |

[a] All genes (*cps2A-2S*) in the cps gene clusters were sequenced.
[b] Inactivating mutation (confirmed by complementation analysis).

**Table 1.8.** Mutations that critically affected the capsule synthesis of *S. suis* isolates found in this study.

| Position of the mutation | Structural alteration | | Single nucleotide substitution | | Frameshift mutation | Total |
|---|---|---|---|---|---|---|
| | Insertion | Deletion | Nonsense | Missense | | |
| In *cps2E-2F* | 7 | 6[a] | 3 | 5[b] | 11 | 32 |
| Out of *cps2E-2F* | 3 | 5 | 0 | 0 | 3[c] | 11 |

[a] Other *cps* genes were also deleted in four of the six isolates (NL204, NL217, NL290, and NL319).

[b] A missense mutation in *cps2R* and an insertion in *cps2K* were also present in one of the five isolates (NL143).

[c] Although one of the three isolates (NL132) had a missense mutation in *cps2E*, the mutation was not involved in the loss of the capsule.

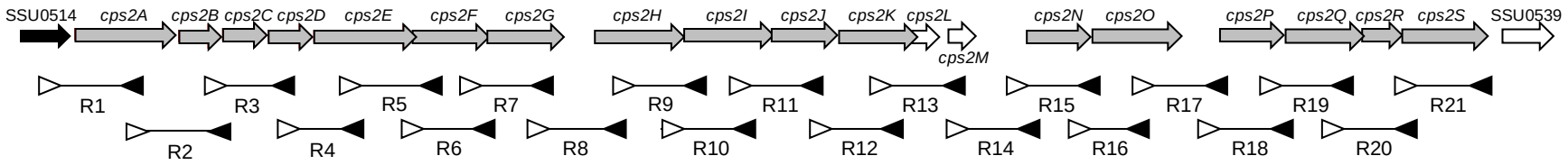



**Figure 1.1.** Genetic organization of serotype 2 *cps* gene cluster (Holden *et al.*, 2009; Okura *et al.*, 2013) and positions of primers used for PCR analysis. Gray arrows represent genes putatively involved in capsule synthesis. Black arrow indicates gene encoding hypothetical protein. Open arrows indicate pseudogenes or genes with unknown functions. Open and closed arrowheads represent forward and reverse primers, respectively. The latest *cps* gene names reassigned by Okura *et al*. (2013) were used in this figure. R, reaction numbers which correspond to the number of primer pairs.

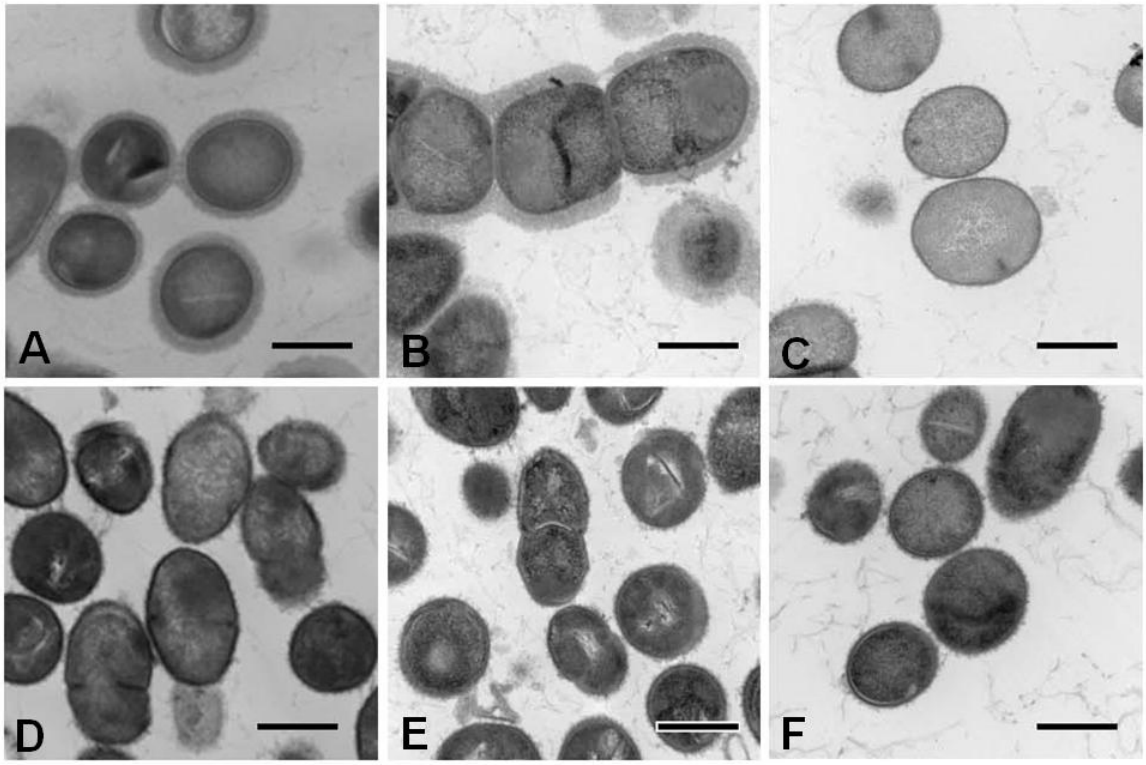



**Figure 1.2.** Transmission electron micrographs of ultrathin sections of *S. suis* strain and isolates. Cells of strain P1/7 (A) and isolate NL333 (B) were obviously surrounded by the capsule, while capsular materials were not seen in NL146 (C), NL194 (D), NL240 (E), and NL290 (F). Bar = 0.5 μm.

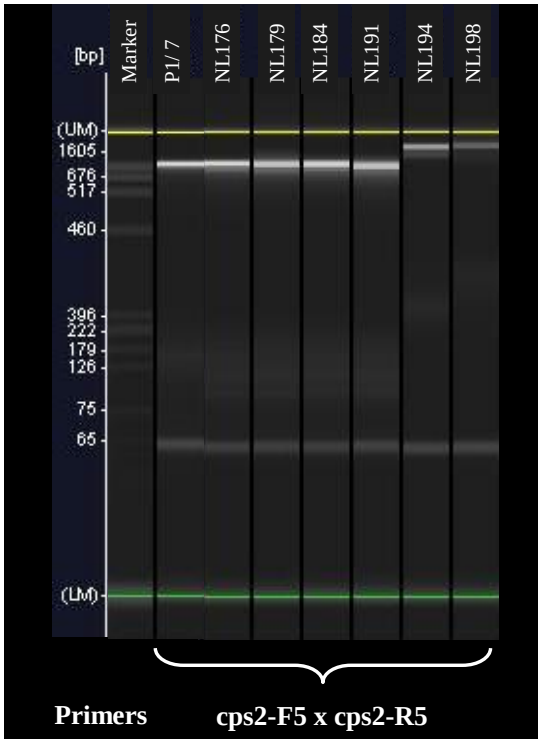



**Figure 1.3.** PCR analysis results of the six representative isolates examined by MultiNA microchip electrophoresis system. DNA extracted from NL176, NL179 and NL184 gave expected PCR amplifications, while the amplified product from NL191 was slightly smaller and the products from NL194 and NL198 were apparently larger than that amplified from positive control (*S. suis* strain P1/7).

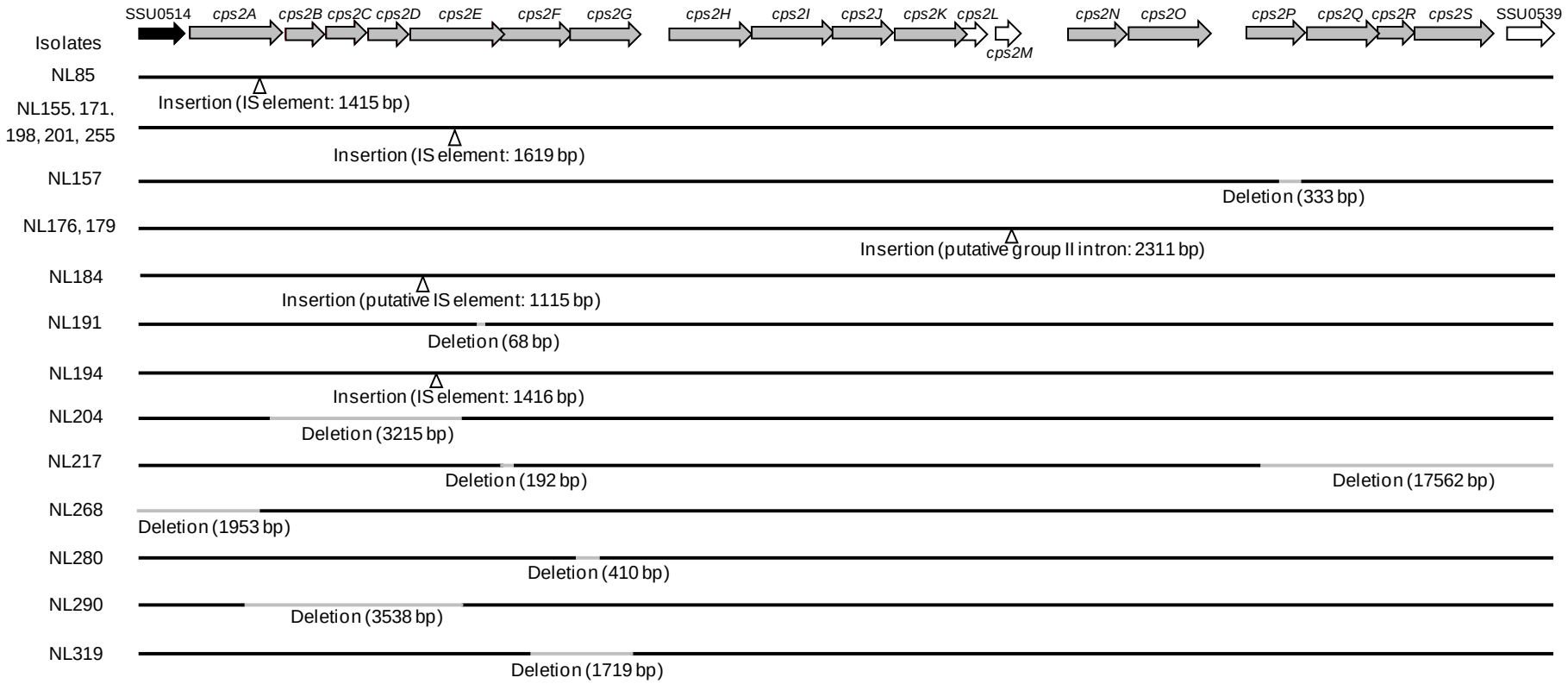



**Figure 1.4.** Positions and types of structural alteration occurred in the *cps2* gene clusters of representative unencapsulated endocarditis isolates. Gray arrows represent genes putatively involved in capsule synthesis. Black arrow indicates gene encoding hypothetical protein. Open arrows indicate pseudogenes or genes with unknown functions. Open triangles indicate positions of insertion. Gray lines indicate deleted regions.

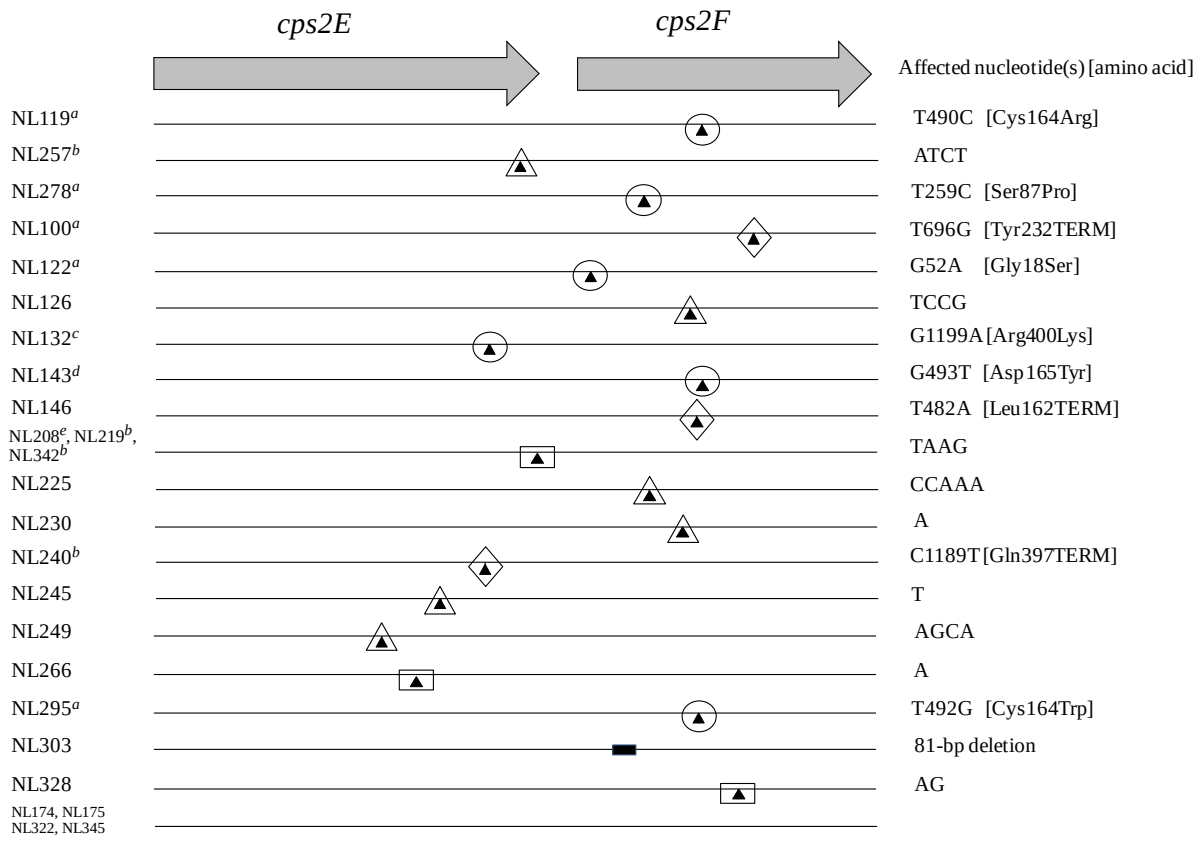



**Figure 1.5.** Sequencing results of genes in *cps2EF* region of 25 unencapsulated isolates. Closed triangles indicate the positions of mutations. Open circles and diamonds indicate missense and nonsense mutations, respectively. Open triangles and open rectangles indicate frame shift mutations caused by insertions and deletions, respectively. The closed rectangle represents the deleted region. [a] The isolates could be complemented with pCps2F. [b] The isolates could be complemented with pCps2E. [c] The isolate could not be complemented with pCps2E. [d] The isolate could not be complemented with pCps2F. [e] Because no transformant was obtained, complementation of *cps2E* could not be accomplished.

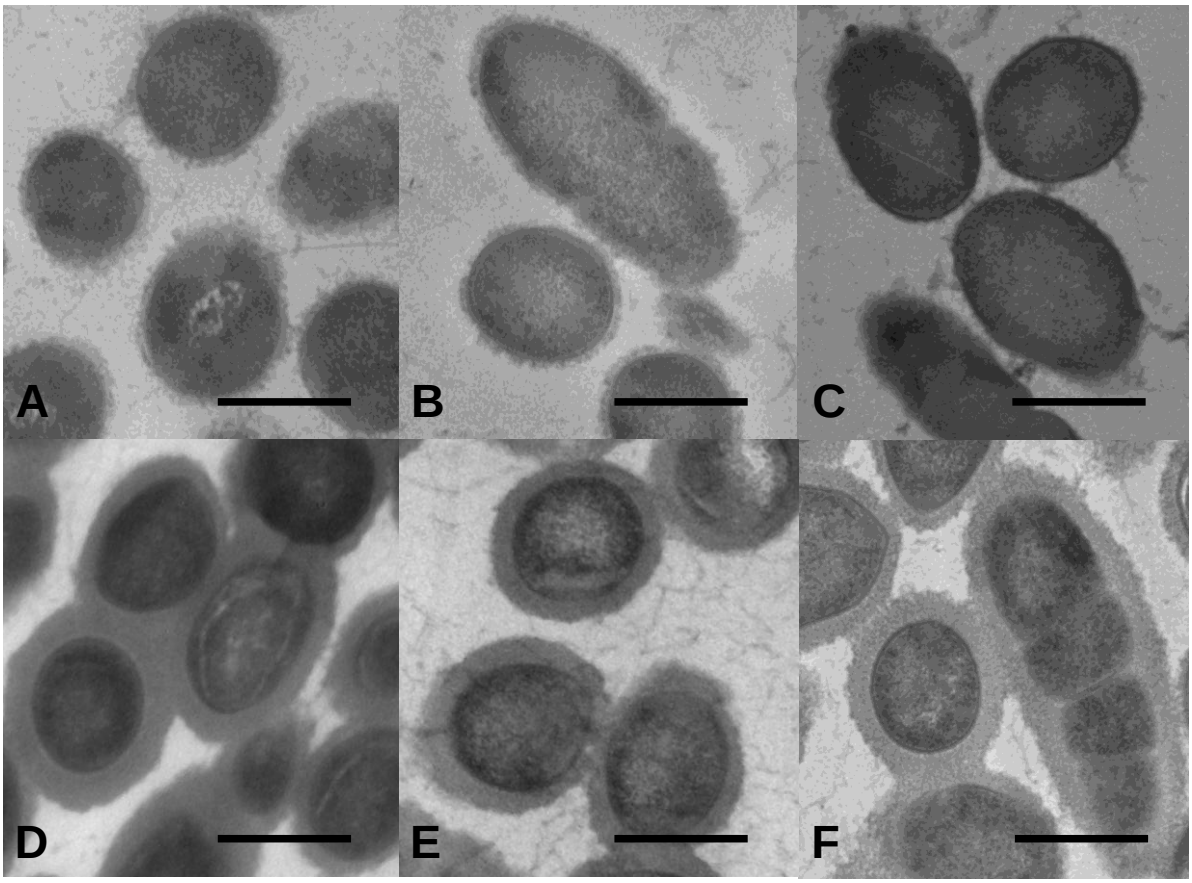



**Figure 1.6.** Transmission electron micrographs of *S. suis*. Ultrathin sections of coagglutination-negative isolates [(A) NL119, (B) NL257, and (C) NL278] and their complemented mutants [(D) NL119(pCps2F), (E) NL257(pCps2E), and (F) NL278(pCps2F)] were observed. Bar = 0.5 μm.

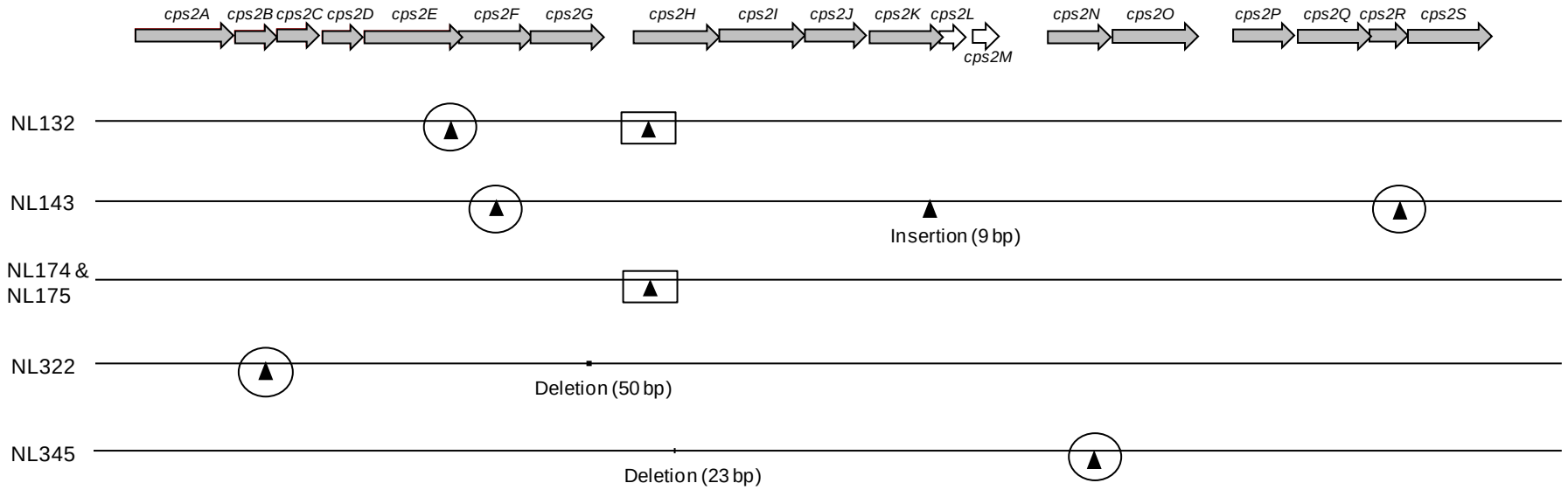



**Figure 1.7.** Putative inactivating mutations in the *cps* gene clusters of six unencapsulated isolates. Closed triangles indicate the positions of mutation. Open circles and rectangles indicate missense and frame shift mutations (caused by deletion), respectively. Black box represents deleted region.

# Chapter 2

# Putative lethal mutation in *cps2* gene cluster of *S. suis*

## 2.1. Introduction

CP of *S. suis* serotype 2 consists of glucose, galactose, *N*-acetyl glucosamine, rhamnose and sialic acid (Elliott & Tai, 1978; Smith *et al.*, 1999a) and is considered to be synthesized by the Wzx/Wzy-dependent pathway. The biosynthesis of CP requires a group of *cps* genes clustered on the chromosome (Okura *et al.*, 2013; Smith *et al.*, 1999a; Smith *et al.*, 2000; Van Calsteren *et al.*, 2010) (Figure 2.1). Among the genes in the cluster, *cps2J* is served as a molecular marker for serotypes 2 and 1/2, and thus, *cps2J*-positive strains are suspected to have capsule of serotype 2 or 1/2 (Smith *et al.*, 1999c). However, the results from chapter 1 demonstrated that 34% of *cps2J*-positive *S. suis* isolates from porcine endocarditis have lost their capsules. Of 43 representative unencapsulated isolates analyzed by PCR analysis, 18 isolates had structural alterations in their *cps* gene clusters. Among them, 16 isolates had deletion or insertion within *cps2A-2G* and *cps2P-2S* (*neuBCDA*) regions of their *cps* gene clusters. The sequencing and complementation results of *cps* genes in the remaining 25 isolates demonstrated that more than 70% of the representative unencapsulated isolates have lost their capsules because of the mutations in *cps2EF* region. On the other hand, the mutation within the *cps2IJ* and *cps2NO* regions was rarely found in the unencapsulated endocarditis isolates analyzed in this study. Although a missense mutation was found in *cps2N* of NL345, this isolate also had a 23-bp deletion in *cps2H* (Table 1.7). Consequently, the mutations in the *cps2IJ* and *cps2NO* regions were proposed to affect the viability of *S. suis*, and mutations in these regions may be restricted only to the isolate carrying the additional mutation in other *cps* gene(s).

The objective of the present chapter is to clarify whether the mutations in the *cps2IJ* and *cps2NO* regions are lethal for *S. suis* by the attempts to construct various *cps* deletion mutants.

## 2.2. Materials and methods

### 2.2.1. Bacterial strains and growth conditions

Bacterial strains used in this chapter are listed in Table 2.1. *S. suis* strains were grown overnight in THB or THA (Difco Laboratories) at 37ºC in air plus 5 % $CO_2$, unless otherwise indicated. *E. coli* strain was cultured in Luria-Bertani (Difco Laboratories) broth or agar at 37ºC. When required, spectinomycin was added to the medium for *E. coli* at 50 µg/ml and for *S. suis* at 100 µg/ml. If necessary, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside was added to the plates at 100 µg/ml.

### 2.2.2. Plasmids, primers, and DNA techniques

Plasmids used for the experiments in this chapter are listed in Table 2.1. Genomic DNA of *S. suis* was extracted as described previously (Mogollon *et al.*, 1990), and plasmid DNA of *E. coli* was purified as described in chapter 1. Primers listed in Table 2.2 and iProof HF Master mix (Bio-Rad Laboratories) were used for PCR to construct *cps* gene knockout vectors. The purification of PCR product, sequencing and analysis of the sequences were performed as described in chapter 1. Restriction enzymes and DNA-modifying enzymes were purchased from Takara Bio and used according to the manufacturer's recommendations. The transformations of *E. coli* and *S. suis* were carried out following the procedures described in chapter 1.

### 2.2.3. Construction of double crossover Δ*cps2EF*, Δ*cps2IJ* and Δ*cps2NO* mutants

The flanking regions of *cps2EF*, *cps2IJ* and *cps2NO* were amplified and fused by overlap-extension PCR (Warrens *et al.*, 1997) using the primers listed in Table 2.2. The resultant PCR products were digested by PstI, EcoRI, and BamHI, respectively, and directly cloned into the respective sites of the temperature-sensitive *S. suis*-*E. coli* shuttle vector

pSET4s (Takamatsu *et al.*, 2001a) to generate *cps2EF*, *cps2IJ*, and *cps2NO* knockout vectors. After introducing *E. coli* TOP10, the sequences of the knockout vectors were verified, and each knockout vector was subsequently introduced into *S. suis* strains 89/1591 and 89/1591Δ*cps2EF* by electroporation. Single crossover mutants were obtained by shifting the incubation temperature of the transformants to 37ºC in the presence of spectinomycin. The single crossover mutants obtained were then subcultured several times in THB at 28ºC in the absence of antibiotics, and dilutions of the subculture were plated on nonselective THA plates. The colonies that formed after overnight culture of the plates were screened for loss of vector-mediated spectinomycin resistance by inoculating these colonies to both THA–spectinomycin and nonselective plates, and spectinomycin-susceptible colonies were selected as candidates, which may lose the target *cps* genes or returned to the wild-type as a result of double crossover recombination event. The deletion of target genes was confirmed by PCR, and if necessary, by sequencing.

## 2.3. Results

### 2.3.1. Generation of knockout mutants under wild-type and Δ*cps2EF* backgrounds

For verification of the hypothesis that mutations in *cps2IJ* and *cps2NO* regions may affect the viability of *S. suis* and that the mutation in these regions may occur only in the presence of other mutation in *cps* gene(s), *cps2IJ* and *cps2NO* knockout vectors were introduced to strain 89/1591, generated single crossover mutants, in which the knockout vectors were integrated into the chromosome (Figure 2.2), and investigated whether double crossover Δ*cps2IJ* and Δ*cps2NO* mutants could be obtained from single crossover mutants. In addition, strain 89/1591Δ*cps2EF*, which had lost its capsule due to the deletion of *cps2EF*, was also used as a parent strain to construct Δ*cps2IJ* and Δ*cps2NO* mutants. As illustrated in Figure 2.2, after the single crossover event between the introduced vector and host

chromosome, two types of single crossover mutants (pattern 1 and 2 mutants) were generated. However, when the *cps2IJ* knockout vector was used, only pattern 2 single crossover mutant was obtained from strain 89/1591. Therefore, only pattern 2 mutants were used to construct double crossover Δ*cps2IJ* mutants.

The numbers of double crossover Δ*cps2IJ* and Δ*cps2NO* mutants obtained from strains 89/1591 and 89/1591Δ*cps2EF* are summarized in Table 2.3. Although 2,556 and 16 double crossover recombinants were obtained from strain 89/1591 transformed with *cps2IJ* and *cps2NO* knockout vectors, respectively, all the recombinants tested turned back to the parent genotype via double crossover recombination, and neither the Δ*cps2IJ* nor Δ*cps2NO* mutant was obtained. However, when 89/1591Δ*cps2EF* was used as the host strain, Δ*cps2IJ* and Δ*cps2NO* mutants were obtained in 31.8% and 62.5%, respectively, of double crossover recombinants.

## 2.4. Discussion

Using molecular approaches, the results from the previous chapter showed that more than 70% of unencapsulated *S. suis* clinical isolates have lost their capsule because of inactivating mutations related to *cps2E* and *cps2F*, whereas no inactivating mutation was detected in *cps2I*, *cps2J*, or *cps2O* and only one missense mutation was found in *cps2N* among the unencapsulated endocarditis isolates analyzed in this study. Consistent with this result, experiments using recombination techniques suggested that the deletion of the *cps2IJ* and *cps2NO* regions was lethal for *S. suis*. *S. suis* CP is considered to be synthesized via Wzx/Wzy dependent pathway. In this pathway, as shown in Figure 2.1B, the initial glycosyltransferase catalyzes the transfer of the first sugar to a membrane-associated lipid carrier, and then further glycosyltransferases sequentially link additional sugars to form repeat units on the inner face of the cytoplasmic membrane. The Wzx flippase subsequently

transports the single repeat unit across the cytoplasmic membrane, and Wzy polymerase polymerizes the individual repeat units to generate lipid-linked CPs. Finally, mature CP is translocated to the peptidoglycan by the membrane protein complex encoded in the *cps* gene cluster (Bentley *et al.*, 2006). According to the CP structure and proposed functions of *cps* genes of *S. suis* serotype 2, *cps2E* and *cps2F* are considered to encode the initial and second glycosyltransferases, respectively, of the Wzx/Wzy dependent pathway, whereas *cps2J* and *cps2N* are thought to be involved in side chain formation of the repeat unit (Figure 2.1B) (Van Calsteren *et al.*, 2010). In addition, *cps2I* (*wzy*) and *cps2O* (*wzx*) were proposed to be responsible for capsular polysaccharide polymerase and flippase, respectively (Okura *et al.*, 2013; Smith *et al.*, 1999a; Smith *et al.*, 2000). Therefore, in *S. suis*, mutations of the genes involved in CP backbone formation seem to cause capsule loss, while loss of function of the genes responsible for the side chain formation, polymerase, and flippase are likely to be deleterious for *S. suis*.

In *Streptococcus pneumoniae* serotype 2, the CP of which is synthesized by Wzx/Wzy dependent pathway, mutations in *cps* genes involved in the side chain formation, polymerase, and flippase were also suggested to be lethal (Xayarath & Yother, 2007). In the Wzx/Wzy dependent pathway, the undecaprenyl-phosphate (Und-P) is utilized as an essential lipid carrier molecule. The CP biosynthesis initiates on Und-P, and after transferring the repeat unit or polymer of CP to the final acceptor, Und-P is reversibly transported into the cytoplasmic membrane for recycling. In addition to initiation of the CP synthesis, Und-P is also used for the synthesis of peptidoglycan and teichoic acids in Gram-positive bacteria (Yother, 2011). Thus, lethality of mutations within these genes in *S. pneumoniae* is considered to result from sequestration of Und-P in the incomplete capsule synthesis pathway, reduction of available Und-P, and impairment of Und-P utilization in other crucial pathways (Xayarath & Yother, 2007; Yother, 2011). Moreover, the lethality of these

mutations is also possibly due to the accumulation of lipid-linked intermediates or single repeat units on the inner face of the cytoplasmic membrane, resulting in destabilization of the membrane (Xayarath & Yother, 2007; Yother, 2011). Because *S. suis* and *S. pneumoniae* utilize very similar CP biosynthesis pathways; therefore, a same explanation can reasonably describe the lethality in *S. suis*. Interestingly, deletions of genes involved in side chain formation, polymerase, and flippase occurred in the presence of additional mutations in *S. pneumoniae*, which were mostly located in the initial glycosyltransferase gene (Xayarath & Yother, 2007). Similar to this, although the deletion of *cps2IJ* and *cps2NO* regions did not occur in the parent *S. suis* strain, Δ*cps2IJ* and Δ*cps2NO* mutants could be obtained in the absence of the *cps2EF* region (Table 2.3), which indicated that mutations in the *cps2EF* region relieved the stress induced by lethal mutations in the *cps2IJ* and *cps2NO* regions. CP biosynthesis in the Δ*cps2EF* mutant is not supposed to start due to lack of the initial glycosyltransferase; therefore, even if the strain has mutations in the *cps2IJ* and *cps2NO* regions, free Und-P may be abundantly present on the cytoplasmic membrane; thus other crucial pathways may proceed smoothly.

Because no transformant appeared from NL208 (chapter 1), complementation analysis of this isolate could not be accomplished. On the basis of the discussion described above, this failure was supposed to occur because of lethal *cps* mutations, i.e., NL208 possesses mutations somewhere in the *cps2IJ* and *cps2NO* regions in addition to the *cps2E* mutation, and the lethal effect of the mutations was invoked after supplying functional *cps2E*. Genes in the *cps2IJ* and *cps2NO* regions of NL208 were subsequently sequenced to investigate this hypothesis, and were shown to be intact; therefore, this difficulty may be due to other factors. Since NL208 exhibits adenine methylation activity (Figure 2.3), factors that protect it from foreign DNA, such as restriction-modification system and clustered regularly interspaced short palindromic repeats, may influence the transformation efficiency of the isolate. On the

other hand, in NL143, which had inactivating mutations in both *cps2F* and *cps2K*, although functional *cps2F* was successfully introduced into the isolate, the transformant showed very poor growth. It was also noted that the transformant introduced by functional *cps2K* was able to grow as well as the parent isolate (Figure 2.4). As illustrated in Figure 2.1B, *cps2K* was putatively involved in the side chain formation of the CPs, which implied that the mutation in *cps2K* may also be harmful to *S. suis* and that the mutation in *cps2F* was necessarily generated to relieve the detrimental effect from the *cps2K* mutation.

According to the results from chapter 1, the mutations were detected in all 43 representative isolates. Moreover, the mutations in *cps* regions were found to cause the loss of capsule or proposed to cause the death in *S. suis*. The lethal effect due to the mutations in genes involved in the side chain formation, polymerase, and flippase may highlight the alternative target for the development of novel therapeutic or preventive strategies for *S. suis* infection.

## 2.5. Summary

In the present chapter, the attempts to construct Δ*cps2IJ* and Δ*cps2NO* mutants have been made via double crossover recombination. Although 2,556 and 16 double crossover recombinants were obtained from strain 89/1591 transformed with *cps2IJ* and *cps2NO* knockout vectors, respectively, all the recombinants tested turned back to parent genotype. However, interestingly, the Δ*cps2IJ* and Δ*cps2NO* mutants can be obtained when 89/1591Δ*cps2EF* was used as a host strain. The results suggested that Δ*cps2IJ* and Δ*cps2NO* mutants can be derived only from the Δ*cps2EF* mutant, but not from its parent strain (89/1591), implying that the mutations in *cps2IJ* and *cps2NO* regions are lethal for *S. suis*, but the mutation in *cps2EF* region is effective in relieving the stress induced by the mutations in the two regions.

**Table 2.1.** Bacterial strains and plasmids used in this study.

| Strain or plasmid | Relevant characteristics | Source or reference |
|---|---|---|
| *S. suis* strains | | |
| 89/1591 | Wild-type strain isolated from a pig with septicemia and meningitis | (Salasia *et al.*, 1995) |
| 89/1591Δ*cps2EF* | 89/1591 derivative carrying a deletion in *cps2E-cps2F* | This study |
| 89/1591Δ*cps2IJ* | 89/1591 derivative carrying a deletion in *cps2I-cps2J* | This study |
| 89/1591Δ*cps2NO* | 89/1591 derivative carrying a deletion in *cps2N-cps2O* | This study |
| *E. coli* strain | | |
| TOP10 | Host for pSET4s derivatives | Invitrogen |
| Plasmids | | |
| pSET4s | $Spc^r$, pUC *ori*, thermosensitive pG+host3 *ori*, *lacZ'* | (Takamatsu *et al.*, 2001a) |
| *cps2EF* knockout vector | pSET4s carrying the flanking regions of *cps2EF* | This study |
| *cps2IJ* knockout vector | pSET4s carrying the flanking regions of *cps2IJ* | This study |
| *cps2NO* knockout vector | pSET4s carrying the flanking regions of *cps2NO* | This study |

**Table 2.2.** Primers used for construction of *cps* knockout vectors.

| Primer | Sequence (5'-3') | Description | PCR program[a] |
|---|---|---|---|
| For construction of *cps2EF* knockout vector | | | |
| 1-cps2EF | GCGACTCTTCGTCGCTATTA | For amplification of upstream region of *cps2E*. | 98°C 2min - 98°C 10sec, 55°C 10sec, 72°C 1min (30 cycles) - 72°C 2min |
| 2-cps2EF | ACCACGTCTTGAAAGTGCGA | For amplification of upstream region of *cps2E*. | |
| 3-cps2EF | GGAACGAACCCATCTTTACT | For amplification of downstream region of *cps2F*. | 98°C 2min - 98°C 10sec, 55°C 10sec, 72°C 1min (30 cycles) - 72°C 2min |
| 4-cps2EF | ACTTTGAGGGAGGTGTAGAC | For amplification of downstream region of *cps2F*. | |
| 5-cps2EF | GATTCTGCAGCGAAGCTTATCGTCAAGGTG | Used for amplification of upstream border of *cps2E* and generation of fused fragment to vector. PstI site is underlined. | 98°C 2min - 98°C 10sec, 60°C 10sec, 72°C 1min (30 cycles) - 72°C 2min |
| 6-cps2EF | TTCGCTCCTTCTTCCGCCAATTTCGTTTGG | Used for amplification of upstream border of *cps2E* and generation of fused fragment to downstream-border fragment of *cps2F*. | |
| 7-cps2EF | CCAAACGAAATTGGCGGAAGAAGGAGCGAA | Used for amplification of downstream border of *cps2F* and generation of fused fragment to upstream-border fragment of *cps2E*. | 98°C 2min - 98°C 10sec, 60°C 10sec, 72°C 1min (30 cycles) - 72°C 2min |
| 8-cps2EF | TCTCCTGCAGACCGAGCATCGGACTGA | Used for amplification of downstream border of *cps2F* and generation of fused fragment to vector. PstI site is underlined. | |

[a] All PCRs were performed by using iProof HF Master mix (Bio-Rad Laboratories).

**Table 2.2. (continued)** Primers used for construction of *cps* knockout vectors.

| Primer | Sequence (5'-3') | Description | PCR program[a] |
|---|---|---|---|
| For construction of *cps2IJ* knockout vector | | | |
| 1-cps2IJ | GAGGCATATAATCAGTATCG | For amplification of upstream region of *cps2I*. | 98°C 2min - 98°C 10sec, 55°C 10sec, 72°C 1min (30 cycles) - 72°C 2min |
| 2-cps2IJ | TTGCTGACATATTCCGATAG | For amplification of upstream region of *cps2I*. | |
| 3-cps2IJ | AAGAGGTGCGAGACTTAGGA | For amplification of downstream region of *cps2J*. | 98°C 2min - 98°C 10sec, 55°C 10sec, 72°C 1min (30 cycles) - 72°C 2min |
| 4-cps2IJ | AAGCTTCTTTTGCTGTTTGC | For amplification of downstream region of *cps2J*. | |
| 5-cps2IJ | GAAAGAATTCGGCGCTCATTGAATCTGGCG | Used for amplification of upstream border of *cps2I* and generation of fused fragment to vector. EcoRI site is underlined. | 98°C 2min - 98°C 10sec, 60°C 10sec, 72°C 1min (30 cycles) - 72°C 2min |
| 6-cps2IJ | AAGCGATTCATCTCCCACAGAAGCTTTGCC | Used for amplification of upstream border of *cps2I* and generation of fused fragment to downstream-border fragment of *cps2J*. | |
| 7-cps2IJ | GGCAAAGCTTCTGTGGGAGATGAATCGCTT | Used for amplification of downstream border of *cps2J* and generation of fused fragment to upstream-border fragment of *cps2I*. | 98°C 2min - 98°C 10sec, 60°C 10sec, 72°C 1min (30 cycles) - 72°C 2min |
| 8-cps2IJ | GGCAGAATTCGCGATGGTCAGTCATACTAG | Used for amplification of downstream border of *cps2J* and generation of fused fragment to vector. EcoRI site is underlined. | |

[a] All PCRs were performed by using iProof HF Master mix (Bio-Rad Laboratories).

**Table 2.2. (continued)** Primers used for construction of *cps* knockout vectors.

| Primer | Sequence (5'-3') | Description | PCR program[a] |
|---|---|---|---|
| For construction of *cps2NO* knockout vector | | | |
| 1- cps2NO | GCTCAAGTTCAGCAGTGGTA | For amplification of 5' flanking region of *cps2N*. | 98°C 2min - 98°C 10sec, 55°C 10sec, 72°C 1min (30 cycles) - 72°C 2min |
| 2- cps2NO | AAGGGGGATACTGGGGTTTA | For amplification of 5' flanking region of *cps2N*. | |
| 3- cps2NO | ACGTCCCAAATGAGAGGTGC | For amplification of 3' flanking region of *cps2O*. | 98°C 2min - 98°C 10sec, 55°C 10sec, 72°C 1min (30 cycles) - 72°C 2min |
| 4- cps2NO | ATCCTACGCTTGTCCGCTTC | For amplification of 3' flanking region of *cps2O*. | |
| 5- cps2NO | CATCGGATCCTTCATTTGTCCTGTGGCTAC | Used for amplification of 5' flanking region of *cps2N* and generation of fused fragment to vector. BamHI site is underlined. | 98°C 2min - 98°C 10sec, 60°C 10sec, 72°C 1min (30 cycles) - 72°C 2min |
| 6- cps2NO | GGCAGTGATGGTCAAGGCATCTGCTAATCT | Used for amplification of 5' flanking region of *cps2N* and generation of fused fragment to 3' flanking fragment of *cps2O*. | |
| 7- cps2NO | AGATTAGCAGATGCCTTGACCATCACTGCC | Used for amplification of 3' flanking region of *cps2O* and generation of fused fragment to 5' flanking fragment of *cps2N*. | 98°C 2min - 98°C 10sec, 60°C 10sec, 72°C 1min (30 cycles) - 72°C 2min |
| 8- cps2NO | TGGCGGATCCCTGTGCTAATCAAGAAGTCC | Used for amplification of 3' flanking region of *cps2O* and generation of fused fragment to vector. BamHI site is underlined. | |

[a] All PCRs were performed by using iProof HF Master mix (Bio-Rad Laboratories).

**Table 2.3.** Numbers of Δ*cps2IJ* and Δ*cps2NO* double crossover mutants derived from the 89/1591 and 89/1591Δ*cps2EF* strains.

| Host strain | Target genes | No. of Δ*cps2IJ* or Δ*cps2NO* mutants/ double crossover recombinants (%) |
|---|---|---|
| 89/1591 | *cps2IJ* | 0/300[a,b] (0%) |
| 89/1591Δ*cps2EF* | *cps2IJ* | 7/22[a] (31.8%) |
| 89/1591 | *cps2NO* | 0/16[c] (0%) |
| 89/1591Δ*cps2EF* | *cps2NO* | 35/56[c] (62.5%) |

[a] Double crossover recombinants were obtained by screening 3000 colonies derived from pattern 2 single crossover mutant (Figure 2.2A).

[b] Of the 2,556 double crossover recombinants obtained, 300 colonies were representatively examined by PCR.

[c] Double crossover recombinants were obtained by screening 2000 colonies from pattern 1 and 2000 colonies from pattern 2 single crossover mutants (Figure 2.2B).

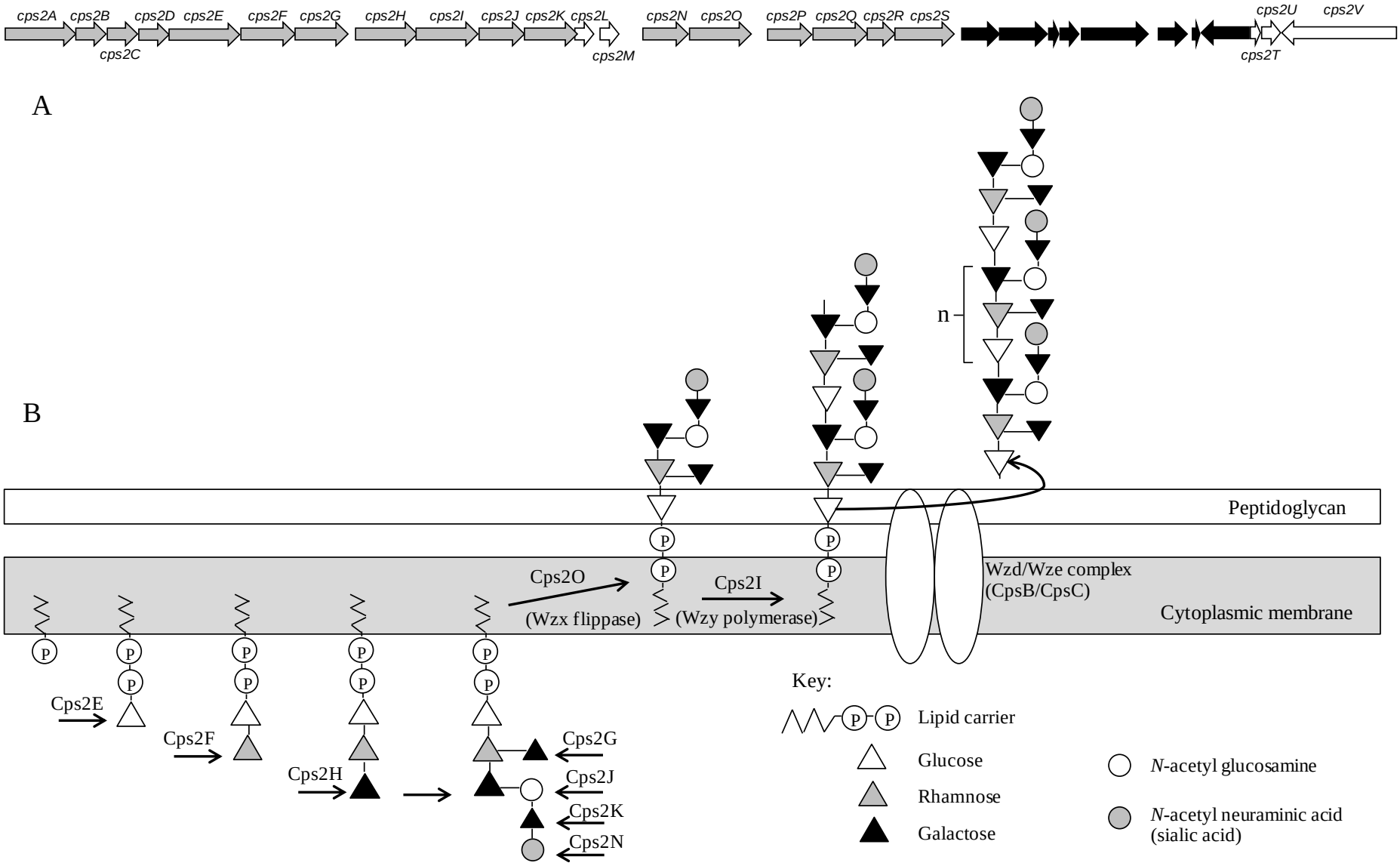
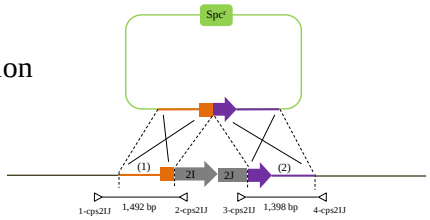
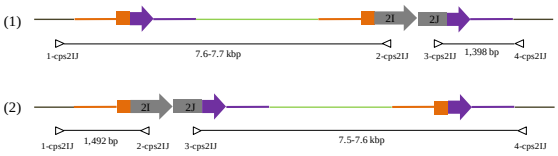

cps2A
cps2B
cps2C
cps2D
cps2E
cps2F
cps2G
cps2H
cps2I
cps2J
cps2K
cps2L
cps2M
cps2N
cps2O
cps2P
cps2Q
cps2R
cps2S
cps2U
cps2T
cps2V
A
B
n
Peptidoglycan
Wzd/Wze complex
(CpsB/CpsC)
Cytoplasmic membrane
Cps2O
(Wzx flippase)
Cps2I
(Wzy polymerase)
Cps2E
Cps2F
Cps2H
Cps2G
Cps2J
Cps2K
Cps2N
Key:
Lipid carrier
Glucose
Rhamnose
Galactose
N-acetyl glucosamine
N-acetyl neuraminic acid
(sialic acid)

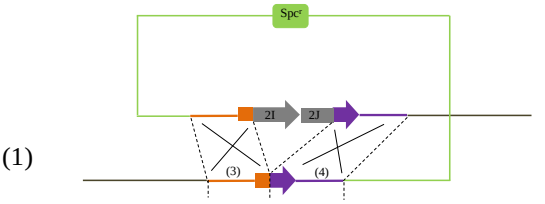
**Figure 2.1.** Genetic organization of *S. suis* serotype 2 *cps* gene cluster and tentative serotype 2 CP biosynthesis model. (A) The genetic organization of serotype 2 *cps* gene cluster (Okura *et al*., 2013). Gray arrows represent genes putatively involved in capsule synthesis. Open arrows indicate genes with unknown function. Black arrows indicate putative transposase or integrase. (B) The hypothetical model for biosynthesis of *S. suis* serotype 2 CPs was illustrated on the basis of the Wzx/Wzy dependent pathway for the biosynthesis of pneumococcal CP proposed by Bentley *et al*. (2006), CP structure of *S. suis* serotype 2, and function of glycosyltransferases proposed by Van Calsteren *et al.* (2010). The putative glucosyl-1-phosphate transferase encoded by *cps2E* transfers the glucose residue to the lipid carrier and then more glycosyltransferases link the additional sugars to form repeat unit. The synthesized repeat unit is transported across the cytoplasmic membrane by the action of flippase encoded by *cps2O* and each repeat unit is polymerized by Wzy polymerase encoded by *cps2I*. Subsequently, mature CP is translocated to the peptidoglycan by the Wzd/Wze protein complex putatively encoded by *cps2B* and *cps2C*. The latest *cps* gene names reassigned by Okura *et al.* (2013) were used in this figure.

A

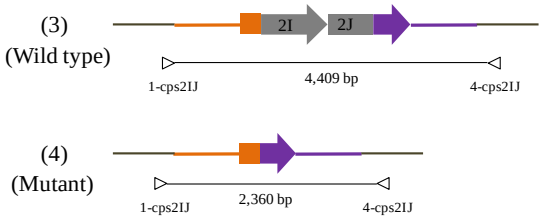
Single crossover recombination



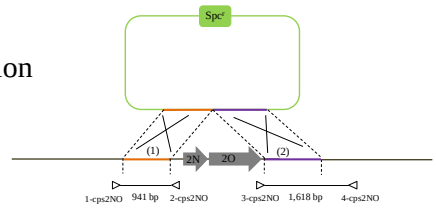



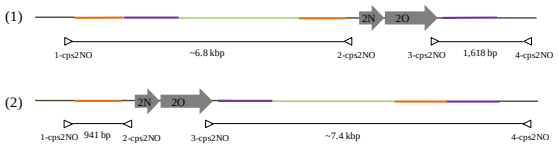

Double crossover recombination

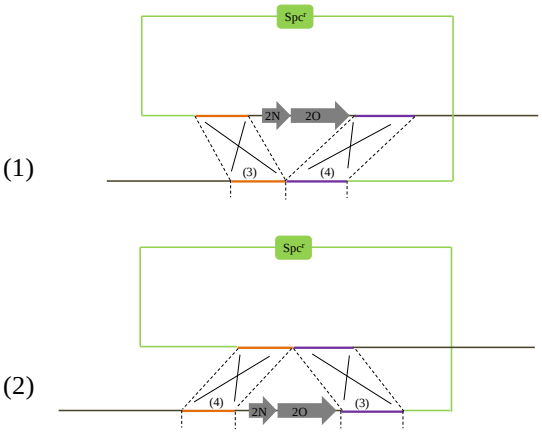



B

Single crossover recombination




Double crossover recombination

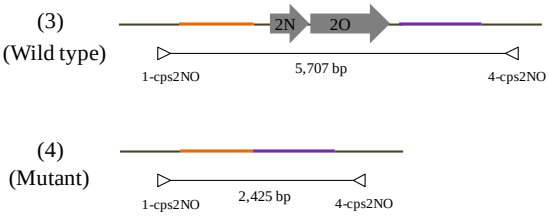


**Figure 2.2.** Patterns of single crossover and double crossover recombination possibly occurred in the course of construction of the Δ*cps2IJ* and Δ*cps2NO* mutants. Open arrow heads represent the positions of primers used for the construction of deletion mutants. The expected size of PCR product was indicated. (A) Patterns of single and double crossover recombination in the construction of Δ*cps2IJ* mutant. (B) Patterns of single and double crossover recombination in the construction of Δ*cps2NO* mutant.


**Figure 2.3.** Methylation of 5′-GATC-3′ sequence in the DNA of unencapsulated isolate NL208. DNA from NL208 was treated with two complementary restriction endonucleases and analyzed by agarose gel electrophoresis. The result showed that DNA of NL208 was digested by DpnI (specific for methylated sequence) but not by MboI (specific for unmethylated sequence), indicating methylation of adenine at its 5′-GATC-3′ sequence.

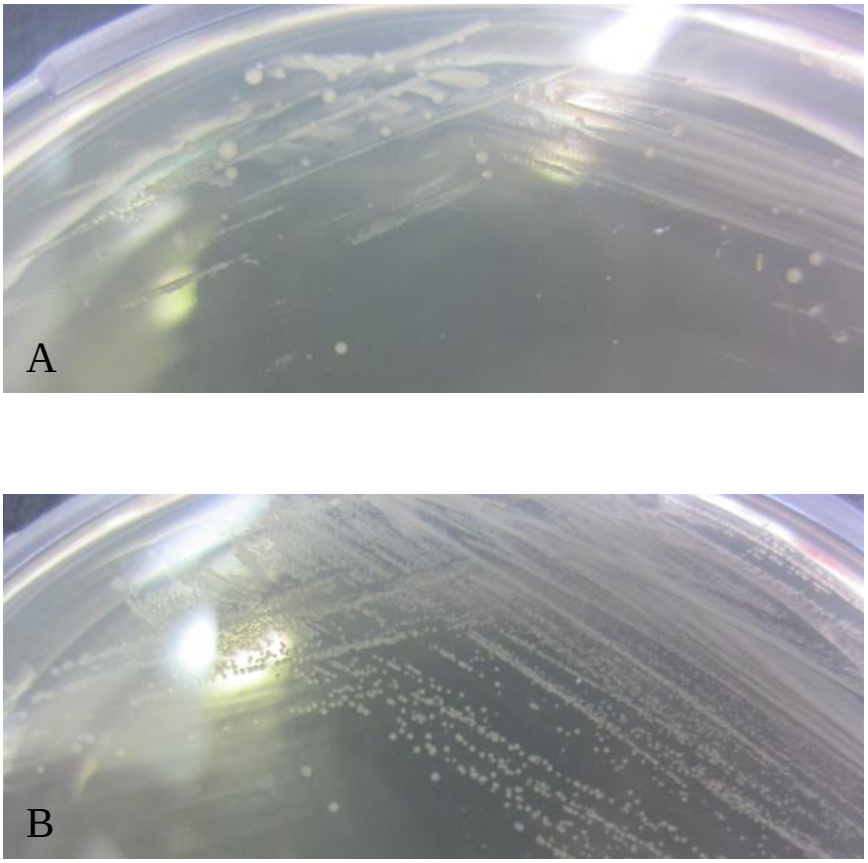



**Figure 2.4.** Growth of the NL143 transformants on THA-spectinomycin plates. (A) NL143 transformant introduced by pCps2F showed very poor growth. (B) NL143 transformant introduced by pCps2K was able to grow well.

# Chapter 3

# The biological significance of the unencapsulation in the pathogenesis of infective endocarditis

## 3.1. Introduction

Bacterial CPs generally serve as a physical barrier to protect bacterial cells from the host immune system. The CPs of *S. suis* have been found to destabilize the lipid microdomains of macrophages and prevent pathogen patterns from being recognized (Houde *et al.*, 2012). Encapsulated *S. suis* has also been proposed to attach to phagocytes and subsequently disseminate through blood circulation leading to septicemia (Segura & Gottschalk, 2002). Moreover, encapsulated *S. suis*, particularly serotype 2, can be viable in dendritic cells, which may enable *S. suis* to be released and then spread in the body to cause an invasive disease (Meijerink *et al.*, 2012). Although capsule has so far been known as an essential virulence factor, the capsule loss in a notable number of *S. suis* isolates from pigs with endocarditis was demonstrated in the previous chapter. According to the results from PCR, sequencing, and complementation analyses, the causes of unencapsulation in all 43 representative isolates were elucidated. However, the phenotypic characteristics which potentially facilitate the pathogenesis of infective endocarditis in these isolates remained unknown.

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). 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 microorganisms 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). As well as the adherence ability, the biofilm plays a key role contributing to the formation of a primary infection lesion on cardiac valve (Parsek & Singh, 2003) and biofilm formation by the bacterial pathogens is also considered to be important for them to persist in host and to protect themselves from host

immune system or to increase the resistance to antibiotics (Donlan & Costerton, 2002). Thus, the isolation of unencapsulated isolates from porcine endocarditis case may result from the advantageous property of the isolates to form septic vegetations.

The aim of this chapter is to examine the biological significance of unencapsulation in the pathogenesis of endocarditis including bacterial adherence to platelets and ability to form biofilm. Furthermore, the ability of encapsulated cells to adhere to the biofilm produced by unencapsulated isolates is investigated and the result suggests further advantage of unencapsulation in the establishment of *S. suis* infection.

## 3.2. Materials and methods

### 3.2.1. Bacterial strains, plasmids and growth conditions

Four unencapsulated *S. suis* isolates and three of their complemented mutants obtained from chapter 1 were selected for the experiments in this chapter. The bacteria and plasmids used in this study are listed in Table 3.1. *S. suis* isolates, strains, and mutants were grown overnight in THB or THA (Difco Laboratories) at 37ºC in air plus 5 % $CO_2$, unless otherwise indicated. *E.coli* strains were cultured in Luria-Bertani (Difco Laboratories) broth or agar at 37ºC. When required, spectinomycin was added to the medium for *E. coli* at 50 µg/ml and for *S. suis* at 100 µg/ml. If necessary, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside was added to the plates at 100 µg/ml.

### 3.2.2. 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 procedure described elsewhere (Hoshinoo *et al.*, 2009) with appropriate modifications. For preparation of the inocula, *S. suis* strains were cultured in THB or THB with spectinomycin (for strain CPS2B) until the absorbance at 600

nm reached 0.8. The cultured bacteria were washed twice with DPBS, sonicated in DPBS for 30 sec to disperse the bacterial cells, and then diluted with DPBS to approximately $2 \times 10^9$ CFU/ml. 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 National Institute of Animal Health (Ibaraki, Japan), 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 prostaglandin $I_2$ 1 μg/ml for first wash), fixed with 0.8 % formalin in DPBS, and immobilized in 8-well culture slides (BD Falcon glass; Becton Dickinson) coated with 0.01 % poly-L-lysine solution at approximately $1 \times 10^8$ platelets/well. The wells were treated with 1 × blocking reagent (Roche Applied Science, Mannheim, Germany) for 1 h with gentle rocking at room temperature to minimize nonspecific adherence. After removal of the blocking reagent by aspiration, 500 μl of each bacterial suspension was inoculated into the wells to give multiplicity of infection at approximately 10. After incubation with gentle shaking for 2 h at room temperature, platelets were washed 4 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 or isolate, the assay was repeated six times using porcine platelets from two different pigs and twelve times using human platelets of three different lots.

### 3.2.3. Construction of double crossover Δ*cps2A-2S* mutant

Double crossover Δ*cps2A-2S* mutant derived from *S. suis* strain P1/7 was constructed to examine the ability to form biofilm. The flanking regions of *cps2A-2S* genes were

amplified by iProof HF Master mix (Bio-Rad Laboratories) in accordance with the manufacturer's instructions using primers listed in Table 3.2 and fused by overlap-extension PCR as described previously (Warrens *et al.*, 1997). The resulting PCR products were cloned into pCR2.1 (Invitrogen, San diego, California, U.S.A.), digested with BamHI and recloned into BamHI site of temperature-sensitive *S. suis* - *E. coli* shuttle vector pSET4s (Takamatsu *et al.*, 2001a) to generate *cps2AS* knockout vector. The double crossover Δ*cps2A-2S* mutant was constructed following the procedures described in chapter 2 and its sequence was subsequently verified.

### 3.2.4. Biofilm formation assay

The biofilm produced by *S. suis* strain P1/7 was compared with that from its isogenic unencapsulated Δ*cps2A-2S* mutant. The production of biofilm was examined as described elsewhere (Grenier *et al.*, 2009) with slight modifications. The overnight cultures of strain P1/7 and its Δ*cps2A-2S* mutant in THB were inoculated to 200 µl of biofilm broth containing (w/v) 0.5% glucose, 2% peptone (proteose peptone no.3), 0.3% $K_2HPO_4$, 0.2% $KH_2PO_4$, 0.01% $MgSO_4 \cdot 7H_2O$, 0.002% $MnSO_4 \cdot 6H_2O$ and 0.5% NaCl in sterile 96-well flat bottom plate and incubated at 37° C for 18-24 h. The medium and free-floating bacteria were gently removed and the wells were washed three times with 200 µl of PBS. After 10 min staining with 100 µl of 0.04% crystal violet, the wells were washed three times with 200 µl of PBS and dried for 2 h at 37 ºC. Then, 100 µl of 95% (v/v) ethanol was added to each well and the plate was shaken for 10 min to release the dye. The biofilms were evaluated by measuring of the absorbance at 570 nm ($A_{570}$). The assay was performed in triplicate and repeated for five times.

### 3.2.5. Adherence of encapsulated cells onto the biofilm produced by unencapsulated isolates

The biofilm of unencapsulated *S. suis* isolates (NL119, NL122 and NL342) was produced by inoculating the isolates into 200 µl of biofilm broth in flat-bottom 96-well plates and incubating the plates at 37° C for 18-24 h. Some wells were incubated with the biofilm broth only, as negative controls.

For the inocula preparation, the encapsulated complemented mutants of NL119, NL122 and NL342 carrying spectinomycin-resistant gene on the complementation vectors were cultured on THA-spectinomycin plates. After overnight incubation, the encapsulated cells were harvested, washed once with THB, and resuspended in THB. The bacterial suspension was diluted with THB until the absorbance at 600 nm reached 0.4, sonicated for 30 sec to disperse the bacterial cells, and then diluted with THB to approximately $1 \times 10^6$ CFU/ml. The bacterial suspensions were additionally diluted in triplicate and each dilution was plated twice onto THA-spectinomycin plates to examine the exact concentration of the inocula each time.

Both biofilm and control wells were gently washed with 100 µl THB followed by 100 µl of each inoculum being inoculated. After incubation with gentle shaking for 2.5 h at room temperature, all wells were washed twice with 100 µl DPBS to remove the unattached cells. Then, 100 µl of fresh THB was added, and the attached cells were retrieved by scraping the bottom of each well and pipetting up and down. The number of attached encapsulated cells was determined by making three independent dilutions and plating 10 µl of each dilution twice on THA-spectinomycin plates. The percentage of encapsulated cells attached to the biofilm was compared with that of the control well. The assay was repeated 5 times in each isolate and the utmost deviate data was excluded from the calculation.

### 3.2.6. Statistical analysis

The results of quantification in each experiment were expressed as means ± standard deviation. The significant differences between the results from encapsulated strain and unencapsulated isolate, strain or negative control were statistically analyzed by the Student's *t*-test at 95% confidence interval ($P < 0.05$).

## 3.3. Results

### 3.3.1. The ability of *S. suis* to adhere to porcine and human platelets

The ability of encapsulated strain P1/7 to adhere to porcine platelets was compared with unencapsulated endocarditis isolate NL194. As shown in Figures 3.1 and 3.2, the average 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) ($P < 0.05$). In addition, the encapsulated serotype 2 strain 89/1591 and its isogenic unencapsulated mutant CPS2B were analyzed to evaluate the effect of unencapsulation. CPS2B also adhered to porcine platelets at greater degree than 89/1591 (average no. of attached bacteria/porcine platelet: 2.02 ± 0.2 for CPS2B vs, 0.44 ± 0.15 for 89/1591; $P < 0.05$) (Figures 3.1 and 3.2). Similar results were obtained when human platelets were used (average no. of attached bacteria/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; $P < 0.05$) (Figure 3.3).

### 3.3.2. Biofilm formation ability of unencapsulated cells

The amount of biofilm produced by encapsulated strain P1/7 was compared to that of its isogenic unencapsulated Δ*cps2A-2S* mutant. As shown in Figure 3.4, the amount of biofilm produced by unencapsulated Δ*cps2A-2S* mutant (biofilm quantification at $A_{570}$: 0.82 ±

0.1) was significantly greater than that of its parent strain P1/7 (biofilm quantification at $A_{570}$: 0.24 ± 0.01), at 95% confidence interval ($P < 0.05$).

### 3.3.3. Effect of biofilm formation by unencapsulated isolates on the adherence of encapsulated cells to surfaces

For demonstrating further advantage of the unencapsulation events, in particular benefit of unencapsulation in a subpopulation to the entire *S. suis* population, the ability of encapsulated *S. suis* cells to adhere to plastic wells coated with and without biofilms of unencapsulated *S. suis* was compared. Three isolates (NL119, NL122, and NL342) and their *cps2E*- or *cps2F*-complemented mutants were used as unencapsulated and encapsulated *S. suis*, respectively.

As shown in Figure 3.5, when NL119 and NL122 were investigated, the ability of encapsulated cells to adhere to biofilms was significantly higher than that to non-coated control wells (% attached encapsulated cells in NL119: 1.36 ± 0.36 for wells with biofilm vs, 0.98 ± 0.28 for control wells; $P < 0.05$ and in NL122: 2.53 ± 0.32 for wells with biofilm vs, 1.18 ± 0.46 for control wells; $P < 0.05$). Although significant differences were not demonstrated due to the unstable results, a similar result was also observed in NL342 (% attached encapsulated cells: 3.28 ± 1.32 for well with biofilm vs, 2.24 ± 1.04 for control well). It was noticeable that NL119 produced less amount of biofilm than NL122 and NL342.

## 3.4. Discussion

Although the advantage of the bacterial capsule has been described so far, Salasia *et al.* (1995) and Benga *et al.* (2005 & 2004) reported that the 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 agreement with the previous studies, the results in this chapter demonstrated that both unencapsulated isolate from endocarditis and the isogenic unencapsulated mutant (CPS2B) had a higher degree of adherence to porcine platelets than the encapsulated strains. Moreover, 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 previously been reported in GBS (Sellin *et al.*, 1992). Therefore, these results may suggest the potential of unencapsulated *S. suis* to cause infective endocarditis in humans.

For elimination of the variation of genetic background and for exact examination of the effect of the unencapsulation on the biofilm formation, an encapsulated *S. suis* strain P1/7 and its Δ*cps2A-2S* mutant were used as the representatives in the biofilm formation assay. Because of the deletion of all *cps* genes putatively involved in CP synthesis, this mutant completely lost its capsule and all components for capsule synthesis. Moreover, the Δ*cps2A-2S* mutant will be useful for the other further studies including the mutagenesis to identify specific genes or proteins involving in the adherence ability. In accordance with the study by Tanabe *et al.* (2010), a capsular-deficient *S. suis* mutant acquired the capacity to form a thick biofilm, which was not observed in the parent strain. Although the exact role of biofilm formation in *S. suis* infections is unclear, such a property may allow bacteria to become

persistent colonizers and to resist clearance by the host immune system. The biofilm-positive phenotype of unencapsulated mutants therefore may give them further advantage to form cardiac vegetations in swine.

As shown in chapters 1 and 2, mutations in the *cps* gene cluster may result in either loss of capsule or death, depending on the position of the mutations. In addition, loss of capsule is known to make the bacterial cells susceptible to phagocytosis by host immune cells; therefore mutations in *cps* genes seem to be risky for individual cells. However, as demonstrated in this study, unencapsulation due to *cps* mutations was actually proved to be beneficial to individual cells in the course of infection by enhancing the ability of the bacterial cells to adhere to host cells as well as to form thick biofilms. Furthermore, the biofilms produced by the unencapsulated cells were demonstrated to help the encapsulated cells to adhere the surfaces. Of note, NL119 produced less amount of biofilm than NL122 and NL342; therefore, this may have caused the relatively lower number of attached encapsulated NL119 mutants to the biofilm compared to the other two.

These results suggest that unencapsulation in *S. suis* can bring an advantage not only to the individual cells but also to the entire population by increasing the adhesiveness of the encapsulated population using the biofilms produced by unencapsulated subpopulation. Therefore, in the host, mutations that occur in *cps* genes may generate unencapsulated subpopulations in the infected *S. suis* population, and such unencapsulated subpopulations may contribute to the establishment of the infection by colonizing on the host surfaces using its increased adherence, forming thick biofilms, and working as a scaffold for further colonization of the remaining encapsulated population.

As shown in the previous chapters, 66% of the endocarditis isolates analyzed in this study were found to have capsule, while 34% were unencapsulated. It is possible that both encapsulated and unencapsulated cells may be present in the same or different steps of the

course of infection; however, both encapsulated and unencapsulated cells have not hitherto be retrieved from the same clinical sample. This is presumably due to a big gap between the numbers of each cell type. Therefore, the additional experiments, e.g. inoculating the experimental animal with both encapsulated and unencapsulated cells and investigating whether both types of cells can be recovered from the infected site in the same animal, should be further performed to verify this hypothesis.

## 3.5. Summary

The binding ability of bacteria to platelets, a major virulence determinant in the pathogenesis of infective endocarditis was analyzed using representative isolate and strains including strain 89/1591, and its isogenic unencapsulated mutant (89/1591CPS2B). The results showed that the platelet-binding ability of unencapsulated isolate and mutant was significantly greater than those of encapsulated strains. Moreover, the unencapsulated cells can enhance the ability to form biofilm and increase the adhesiveness of the strain by increasing the number of the attached encapsulated cells to the biofilm produced at the surface by unencapsulated population. Although the capsule is thought to be an important virulence factor for *S. suis*, these results suggest that the loss of capsular expression is also beneficial for *S. suis* to cause infective endocarditis in swine.

**Table 3.1.** Bacterial isolates, strains, mutants, and plasmids used for the experiments in this chapter.

| Isolate, strain, mutant or plasmid | Relevant characteristics | Source or reference |
|---|---|---|
| *S. suis* isolates, strains, and mutants | | |
| NL119 | Unencapsulated isolate from pigs with endocarditis | This study |
| NL122 | Unencapsulated isolate from pigs with endocarditis | This study |
| NL194 | Unencapsulated isolate from pigs with endocarditis | This study |
| NL342 | Unencapsulated isolate from pigs with endocarditis | This study |
| NL119(pCps2F) | NL119 carrying pCps2F | This study |
| NL122(pCps2F) | NL122 carrying pCps2F | This study |
| NL342(pCps2E) | NL342 carrying pCps2E | This study |
| P1/7 | Wild-type strain isolated from a pig with meningitis | (Slater *et al.*, 2003) |
| 89/1591 | Wild-type strain isolated from a pig with septicemia and meningitis | (Salasia *et al.*, 1995) |
| 89/1591CPS2B | 89/1591 derivative carrying an insertionally inactivated *cps2B* (*cps2B*::pSET4s) | (Okura *et al.*, 2011) |
| P1/7Δ*cps2A-2S* | P1/7 derivative carrying a deletion in *cps2A-2S* genes | This study |
| *E. coli* strain | | |
| TOP10 | Host for pCR2.1 and pSET4s derivatives | Invitrogen |
| Plasmids | | |
| pCR2.1 | $Ap^r$, $Km^r$, pUC *ori*, f1 *ori*, *lacZα* | Invitrogen |
| pSET4s | $Spc^r$, pUC *ori*, thermosensitive pG+host3 *ori*, *lacZ'* | (Takamatsu *et al.*, 2001a) |
| *cps2AS* knockout vector | pSET4s carrying flanking regions of *cps2A-2S* genes | This study |

**Table 3.2.** Primers used for the construction of *cps2AS* knockout vector.

| Primer | Sequence (5'-3') | Description | PCR program[a] |
|---|---|---|---|
| 1-cpsKO | GTTCCTCTATTCCAGTCAAA | For amplification of upstream region of *cps2A*. | 98°C 2min - 98°C 10sec, 55°C 10sec, 72°C 1min (30 cycles) - 72°C 2min |
| 2-cpsKO | ACGACTTACTTCGTCCGCTT | For amplification of upstream region of *cps2A*. | |
| 3- cpsKO | GATGCTAGCCTTGAGAATTG | For amplification of 3' flanking region of *cps2S*. | 98°C 2min - 98°C 10sec, 55°C 10sec, 72°C 1min (30 cycles) - 72°C 2min |
| 4- cpsKO | GTCTACCCACTACAGTTGAC | For amplification of 3' flanking region of *cps2S*. | |
| 5- cpsKO | GCCCAAGCTTATAGACTATGTTACTAGCTA | Used for amplification of 5' flanking region of *cps2A* and generation of fused fragment to vector. HindIII site is underlined. | 98°C 2min - 98°C 10sec, 62°C 10sec, 72°C 1min (30 cycles) - 72°C 2min |
| 6- cpsKO | GACAGCTCTCCTCATACCTATAACGCATCC | Used for amplification of 5' flanking region of *cps2A* and generation of fused fragment to 3' flanking fragment of *cps2S*. | |
| 7- cpsKO | GGATGCGTTATAGGTATGAGGAGAGCTGTC | Used for amplification of 3' flanking region of *cps2S* and generation of fused fragment to 5' flanking fragment of *cps2A*. | 98°C 2min - 98°C 10sec, 62°C 10sec, 72°C 1min (30 cycles) - 72°C 2min |
| 8- cpsKO | ATTGAAGCTTACTAGTTTTTGCCCAGCCTC | Used for amplification of 3' flanking region of *cps2S* and generation of fused fragment to vector. HindIII site is underlined. | |

[a] All PCRs were performed by using iProof HF Master mix (Bio-Rad Laboratories).

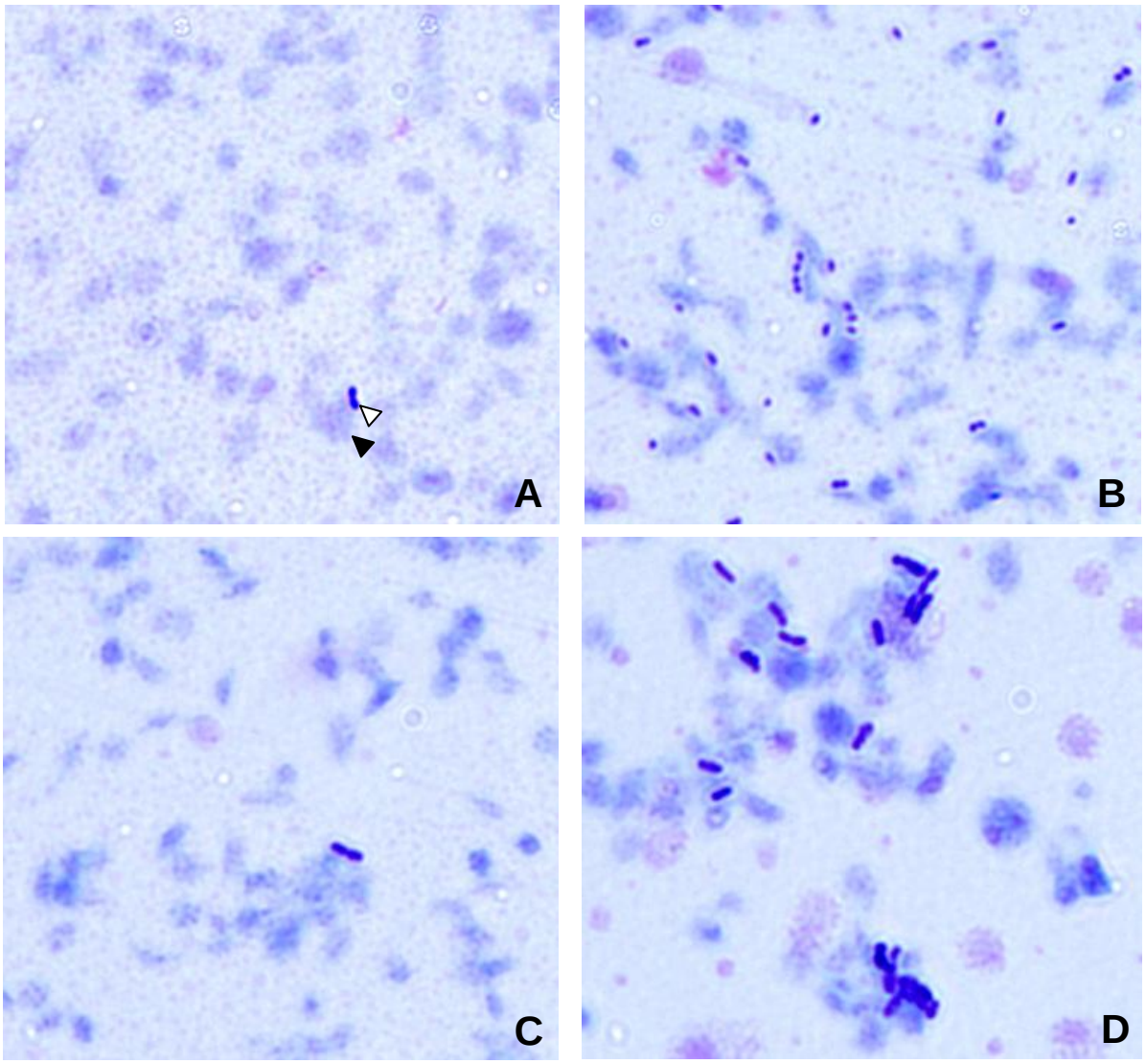



**Figure 3.1.** Adherence of encapsulated and unencapsulated *S. suis* strains, an isolate, and a mutant to porcine platelets. Bacterial cells and platelets were stained with 5 % Giemsa solution and observed under light microscopy: (A) encapsulated serotype 2 strain P1/7; (B) unencapsulated endocarditis isolate NL194; (C) encapsulated serotype 2 strain 89/1591; (D) isogenic unencapsulated mutant of strain 89/1591 (CPS2B). Black and open arrowheads indicate platelets and bacterial cells, respectively. Magnification, 1000x.

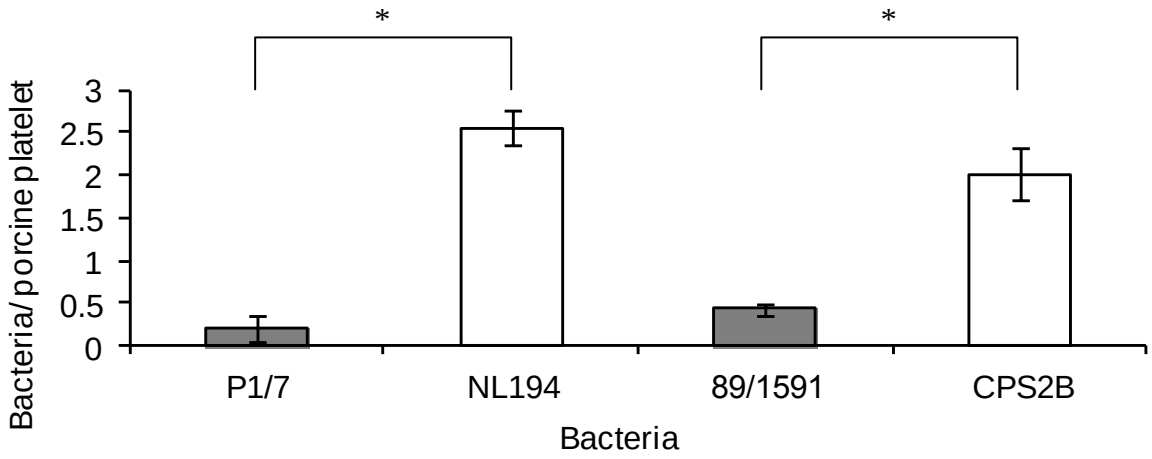



**Figure 3.2.** The ability of *S. suis* strains, an isolate, and a mutant to adhere to porcine platelets. Asterisks indicate significant differences: $P < 0.05$.

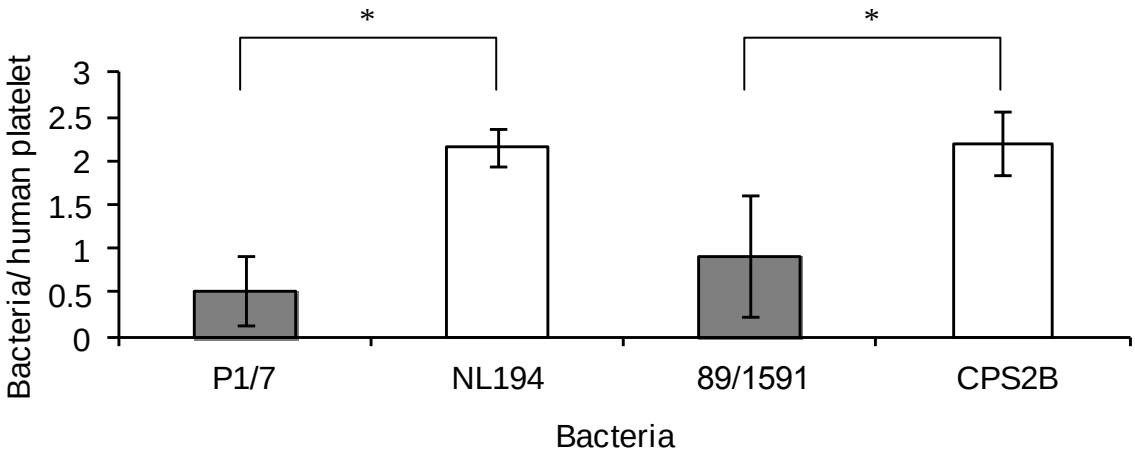



**Figure 3.3.** The ability of *S. suis* strains, an isolate, and a mutant to adhere to human platelets. Asterisks indicate significant differences: $P < 0.05$.

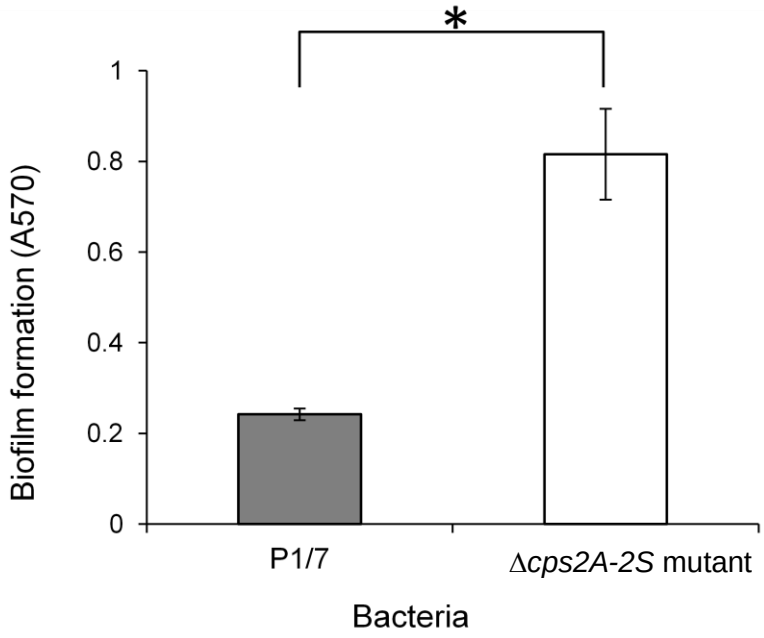



**Figure 3.4.** The biofilm formation ability of *S. suis* strain P1/7 compared with that of its isogenic unencapsulated Δ*cps2A-2S* mutant. Asterisk indicates significant difference: $P < 0.05$.

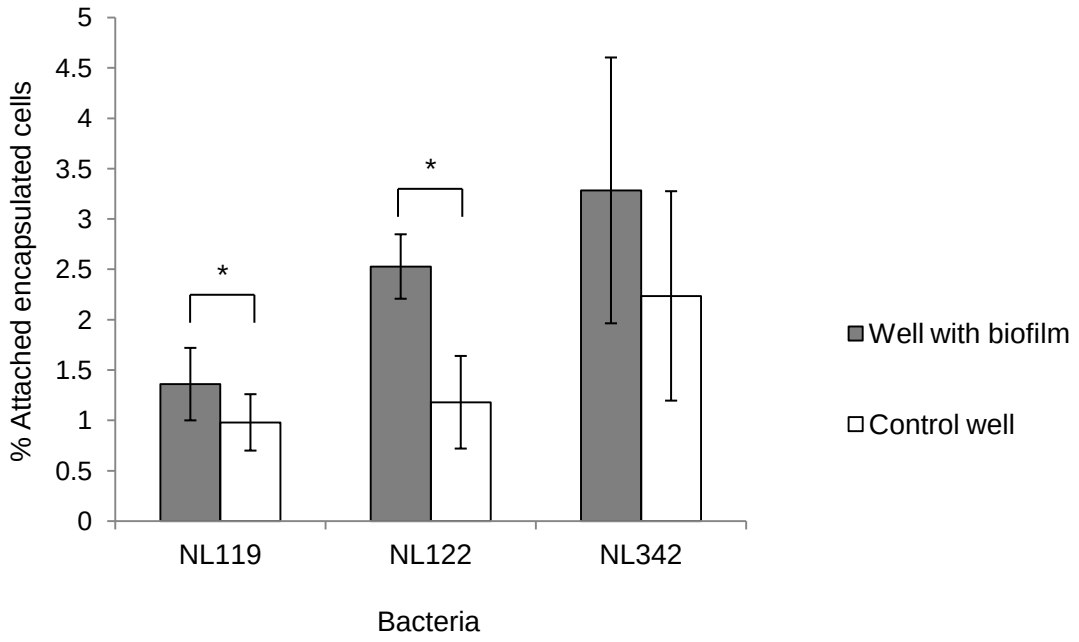



**Figure 3.5.** The adherence of encapsulated cells from complemented mutants to the biofilm produced by their isogenic unencapsulated isolates. The percentage of encapsulated cells attached to biofilm was compared with the negative control (without biofilm formed by unencapsulated cells). Asterisks indicate significant differences: $P < 0.05$.

# General discussion and conclusion

*S. suis*, particularly serotype 2, is a pathogen of both pigs and humans associated with a wide range of diseases, including meningitis, septicemia and endocarditis. CP of serotype 2 consists of glucose, galactose, *N*-acetyl glucosamine, rhamnose, and sialic acid and its biosynthesis requires a group of genes in *cps* gene cluster. Among *S. suis cps* genes, *cps2J* exists only in strains of serotypes 2 and 1/2; therefore, *cps2J*-positive strains are suspected to have capsules of serotype 2 or 1/2. However, it was noticed that, unlike isolates from other symptoms, many *cps2J*-positive isolates from porcine endocarditis were not indeed agglutinated by either anti-serotype 1 or anti-serotype 2 sera, suggesting frequent loss of capsular expression in porcine endocarditis isolates.

The objectives of this study were to verify the loss of capsule in *cps2J*-positive *S. suis* isolates from pigs with endocarditis, to elucidate the unencapsulation mechanism, and to examine the biological significance of the loss of capsule in the pathogenesis of infective endocarditis.

In chapter 1, a total of 288 *cps2J*-positive isolates from diseased pigs were analyzed. By coagglutination tests and TEM, all 32 isolates from pigs with meningitis were confirmed to be encapsulated, while 86 (34%) of 256 isolates from pigs with endocarditis were identified as unencapsulated isolates. Forty-three representative unencapsulated isolates were subsequently selected at random to verify mutations in the genes of *cps* gene cluster by PCR analysis. Among them, 18 isolates were demonstrated to have insertion or deletion(s) in their *cps* gene clusters. Subsequently, the *cps* gene clusters of the remaining 25 isolates were analyzed by sequencing and complementation. The results demonstrated that most of the remaining isolates had nucleotide alterations of a single or a few base pairs in the *cps2E-2F* region, causing frameshift, missense or nonsense mutation(s) of the genes. These results indicated that at least 32 of 43 representative unencapsulated isolates have lost their CPs due to the mutations involving two glycosyltransferase genes (*cps2E* and *cps2F*). On the contrary,

no inactivating mutation was detected in *cps2I*, *cps2J*, and *cps2O* and only one missense mutation was found in *cps2N* among all unencapsulated endocarditis isolates analyzed, implying that mutations in these regions may affect the viability of *S. suis*.

In chapter 2, the attempts to construct various *cps* deletion mutants have been done via double crossover recombination. The results suggested that Δ*cps2IJ* and Δ*cps2NO* mutants can be derived only from the Δ*cps2EF* mutant, but not from its parent strain (89/1591), implying that mutations in *cps2I-2J* and *cps2N-2O* regions are lethal for *S. suis*. However, additional mutations in *cps2E-2F* can relieve the fatal effect induced by the mutations in these two regions.

As well as the majority of *S. pneumoniae* serotypes, *S. suis* CP is considered to be synthesized via Wzx/Wzy dependent pathway. According to the CP structure and proposed functions of *cps* genes of *S. suis* serotype 2, *cps2E* and *cps2F* are considered to encode the initial and second glycosyltransferases, respectively, of the Wzx/Wzy-dependent pathway, whereas *cps2J* and *cps2N* are thought to be involved in side chain formation of the repeat unit. In addition, *cps2I* (*wzy*) and *cps2O* (*wzx*) were proposed to be responsible for capsular polysaccharide polymerase and flippase, respectively. Therefore, in *S. suis* isolates from porcine endocarditis, mutations of the genes involved in CP backbone formation seem to cause their capsule loss, while loss of function of the genes responsible for the side chain formation, polymerase, and flippase are likely to be harmful for *S. suis*.

Since CP has so far been known as an important virulence factor for *S. suis*, the capsule loss of many porcine endocarditis isolates found in this study may suggest some role of unencapsulation in the pathogenesis of endocarditis. Therefore, in chapter 3, the biological significance of unencapsulation to the pathogenesis of infective endocarditis was investigated. The examination of bacterial binding ability to porcine platelets, a major virulence determinant in the pathogenesis of infective endocarditis, using encapsulated strain

P1/7 and a representative unencapsulated endocarditis isolate (NL194) showed that the platelet-binding ability of unencapsulated isolate was significantly higher than that of encapsulated strain. In addition, the unencapsulated mutant 89/1591CPS2B adhered to the platelets greater degree than its encapsulated parent strain 89/1591 ($P < 0.05$). Similar results were also obtained when human platelets were used, suggesting that the frequent isolation of unencapsulated isolates from porcine endocarditis may result from advantageous property of individual unencapsulated cells to cause infective endocarditis.

The biofilm formation ability of the unencapsulated Δ*cps2A-2S* mutant was additionally demonstrated to be significantly higher degree than its encapsulated parent strain P1/7. Although encapsulated *S. suis* strains showed low degree of ability to adhere to host cells, adherence assays using encapsulated and unencapsulated cells demonstrated that the biofilm produced by unencapsulated cells promotes the adherence of encapsulated cells to surfaces. Therefore, in the host, unencapsulated subpopulation generated due to *cps* mutations may contribute to the establishment of the infection by increasing adherence ability to colonize on the host surfaces, along with forming thick biofilm to promote the adhesiveness of the entire population. Although mutations in some *cps* genes were found to be risky for individual cells, these results suggest that loss of capsule production is beneficial for *S. suis* to cause infective endocarditis.

The phenotypic variation derived from genomic variations within bacterial population is generally proposed to be beneficial for the survival of bacteria in stress conditions or environmental changes (Ryall *et al.,* 2012). Genetic heterogeneity may occur during infection in an infected bacterial population, which contributes to its fitness and prolonged existence in the host; therefore, mutations, a major mechanism contributing to the genetic diversity of bacterial populations, may play a crucial role in the course of infections.

As suggested in this study, *cps* mutations may result in the death of individual cells; however, even dead cells are beneficial to the remaining population because they provide public goods for the survivors. By releasing pneumolysin through cell lysis, *S. pneumoniae* can enhance the colonization of its population in the host (Hirst *et al.*, 2004). Extracellular DNA, an important structural component of the *Staphylococcus aureus* biofilm, is also released via cell lysis (Rice *et al.*, 2007). Furthermore, under the condition of amino acid depletion, *E. coli* can trigger programmed cell death, which is proposed to provide nutrients to the remaining cells (Aizenman *et al.*, 1996). Although the advantages obtained from cell death in the *S. suis* subpopulation has not yet been clarified, the results from this study may initially expand the view of altruistic death in the *S. suis* population.

In conclusion, this study demonstrated that approximately one third of the isolates from porcine endocarditis lost the ability to produce capsules and that at least 32 of 43 representative unencapsulated isolates lost their capsule because of the inactivating mutations related to the two glycosyltransferase genes (*cps2E* and *cps2F*). The unencapsulation was further demonstrated to enhance bacterial adherence to both porcine and human platelets and increase the ability to form biofilm. In addition, the data from this study suggested possible advantage of the presence of unencapsulated subpopulations to the entire *S. suis* population. Although it is generally accepted that the loss of the capsule diminishes the virulence of the *S. suis* strain, loss of capsule production is demonstrated to be advantageous for *S. suis* to cause infective endocarditis and further analysis incorporating the findings of this study may provide novel insights into the pathogenic mechanisms of *S. suis* infections. However, because both encapsulated and unencapsulated cells have not confirmed to be retrieved from the same case, the additional experiments are necessarily required for verifying this hypothesis.

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