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Eight Novel Capsular Polysaccharide Synthesis Gene Loci Identified in Nontypeable *Streptococcus suis* Isolates

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***Streptococcus suis* is an important pathogen of pigs and may cause serious disease in humans. Serotyping is one of the important diagnostic tools and is used for the epidemiological study of *S. suis*. Nontypeable *S. suis* strains have been reported in many studies; however, the capsular polysaccharide (CPS) synthesis *cps* loci of nontypeable strains have not been analyzed. In this study, we investigated the genetic characteristics of *cps* loci in 78 nontypeable strains isolated from healthy pigs. Eight novel *cps* loci (NCLs) were found, and all of them were located between the *orfZ-orfX* region and the *glf* gene. All NCLs possess the *wzy* and *wzx* genes, strongly suggesting that the CPSs of these NCLs were synthesized using the Wzx/Wzy-dependent pathway. The *cps* genes found in the 78 isolates were assigned to 96 homology groups (HGs), 55 of which were NCL specific. The encapsulation of the 78 isolates was also examined using transmission electron microscopy. Fifty-three isolates were found to have a capsule, and these were of varied thicknesses. Our data enhance our understanding of the *cps* gene cluster diversity of nontypeable *S. suis* strains and provide insight into the evolution of the *S. suis* capsular genes.**

Streptococcus suis is an important pathogen of pigs (1) and may cause serious disease in humans (2–4). Serotyping is one of the important tools for diagnosis and is used in epidemiological studies of *S. suis*. A total of 35 *S. suis* serotypes (serotypes 1 through 34 and 1/2) are known on the basis of the antigenic differences in their capsular polysaccharides (CPSs) (5–8) and the coagglutination test. The *S. suis cps* gene clusters of the 35 serotypes have been sequenced, and the *cps* gene clusters were shown to be diverse among different serotypes (9). The predicted products of the *cps* genes found in the 35 serotypes were assigned to 291 homology groups (HGs). The precise function of many *cps* genes is still unknown.

The synthesis and export of bacterial polysaccharides are mediated by three pathways known as the Wzx/Wzy-dependent pathway, the synthase-dependent pathway, and the ABC transporter-dependent pathway. The Wzx/Wzy-dependent pathway is most commonly used in *Streptococcus pneumoniae* capsular biosynthesis (10). Although the precise function of most of the *cps* genes of *S. suis* is still unknown, the CPSs of all serotypes of *S. suis* are also thought to be synthesized by the Wzx/Wzy-dependent pathway. This pathway involves the synthesis of the polysaccharide repeat units, which are initially built on the inner face of the cytoplasmic membrane; transport of the repeat units to the outer face of the membrane by Wzx flippase; and then polymerization of the repeat units by Wzy polymerase. Wzy-dependent polymers usually contain various sugars and glycosidic linkages. The specificity of the Wzy polymerase determines the linkage that it catalyzes between sugars on the growing chain and the next repeat unit. Therefore, the *wzy* gene is serotype specific. Hence, the serotype-specific *wzy* gene is an ideal target to discriminate the *cps* loci for molecular serotyping. Recently, two multiplex PCR (mPCR) assays based on the *wzy* gene have been developed to identify *S. suis* serotypes (11, 12). Both methods greatly facilitate the serotype discrimination of *S. suis* isolates.

Clinically healthy pigs can carry *S. suis* in their nasal cavities, tonsils, and upper respiratory tract (13, 14). Nontypeable *S. suis* strains from healthy pigs have been reported in many studies (12, 13, 15–18). The nontypeability of some of these strains is likely due to nonfunctional *cps* loci which produce an acapsular phenotype. However, nontypeable strains may carry at their *cps* loci novel *cps* genes expressing a so far unidentified capsule type. In this study, we obtained the complete sequences of the *cps* loci from 78 nontypeable isolates through Illumina sequencing and primer walking. Eight novel *cps* loci (NCLs), designated NCL1 to NCL8, were identified on the basis of the specific polysaccharide polymerase gene *wzy*. The capsule phenotype of these 78 isolates was also investigated by transmission electron microscopy.

MATERIALS AND METHODS

Bacterial strains and chromosomal DNA preparation. Of 179 *S. suis* field isolates recovered from the tonsils of healthy pigs in 2011 and 2012 in a previous study, 96 isolates were unable to be assigned to a known serotype (19). Seventy-eight of these nonserotypeable isolates were investigated in this study (Table 1). Chromosomal DNA was prepared from all isolates using the method described previously (11). Strains were tested as

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TABLE 1 Isolates used in the study

Isolate	*cps* locus type	Seroagglutination test result	Capsule thickness (nm)	Yr of isolation	Geographic location	MCG group[a]	MLST[b]	*cps* GenBank accession no.
YS7	NCL1-6	Polyagglutinating	No capsule	2011	Beijing	6	ST305	KM972281
YS8	NCL1-6	Polyagglutinating	No capsule	2011	Beijing	6	ST305	KM972290
YS21	NCL1-3	Nonagglutinating	40–60	2011	Sichuan Province	6	ST310	KM972259
YS22	NCL1-3	Nonagglutinating	40–60	2011	Sichuan Province	6	ST310	KM972260
YS23	NCL2-2	Polyagglutinating	30–50	2011	Sichuan Province	6	ST328	KM972261
YS27	NCL3-1	Polyagglutinating	20–40	2011	Beijing	6	ST312	KM972262
YS33	NCL4	Autoagglutinating	No capsule	2011	Beijing	7-3	ST423	KM972263
YS34	NCL4	Polyagglutinating	No capsule	2011	Beijing	7-3	ST314	KM972264
YS35	NCL5	Polyagglutinating	40–60	2011	Beijing	7-2	ST315	KM972265
YS37	NCL4	Autoagglutinating	20–40	2011	Beijing	7-3	ST339	KM972266
YS38	NCL5	Polyagglutinating	30–40	2011	Beijing	7-2	ST315	KM972267
YS41	NCL1-1	Polyagglutinating	No capsule	2011	Beijing	N	ST340	KM972268
YS42	NCL1-4	Nonagglutinating	No capsule	2011	Jiangxi Province	6	ST341	KM972269
YS43	NCL6	Nonagglutinating	No capsule	2011	Jiangxi Province	7-3	ST317	KM972270
YS46	NCL7-2	Polyagglutinating	No capsule	2011	Sichuan Province	6	NT	KM972271
YS49	NCL1-3	Nonagglutinating	No capsule	2011	Sichuan Province	6	ST329	KM972272
YS56	NCL1-1	Polyagglutinating	30–50	2011	Sichuan Province	7-2	ST331	KM972273
YS57	NCL7-1	Polyagglutinating	20–40	2011	Sichuan Province	6	ST320	KM972274
YS68	NCL1-1	Polyagglutinating	40–60	2011	Beijing	6	ST426	KM972275
YS71	NCL1-1	Polyagglutinating	40–60	2011	Jiangsu Province	6	ST347	KM972276
YS72	NCL2-2	Nonagglutinating	40–60	2011	Jiangsu Province	6	ST332	KM972277
YS73	NCL4	Autoagglutinating	70–90	2011	Beijing	7-3	ST348	KM972278
YS74	NCL3-1	Polyagglutinating	20–30	2011	Beijing	6	ST333	KM972279
YS75	NCL3-1	Polyagglutinating	No capsule	2011	Beijing	6	ST349	KM972280
YS80	NCL1-1	Polyagglutinating	40–60	2012	Beijing	6	ST429	KM972282
YS81	NCL1-2	Polyagglutinating	30–50	2012	Jiangsu Province	6	ST398	KM972283
YS82	NCL1-2	Polyagglutinating	30–50	2012	Jiangsu Province	6	ST430	KM972284
YS83	NCL1-2	Polyagglutinating	30–50	2012	Jiangsu Province	6	ST430	KM972285
YS84	NCL1-2	Polyagglutinating	30–50	2012	Jiangsu Province	6	ST398	KM972286
YS85	NCL8-1	Nonagglutinating	50–70	2012	Jiangsu Province	6	ST399	KM972287
YS86	NCL4	Autoagglutinating	20–30	2012	Jiangsu Province	7-3	ST400	KM972288
YS87	NCL8-1	Polyagglutinating	No capsule	2012	Jiangsu Province	6	ST431	KM972289
YS91	NCL3-1	Polyagglutinating	20–30	2012	Jiangsu Province	6	ST451	KM972291
YS92	NCL3-1	Polyagglutinating	20–30	2012	Jiangsu Province	6	ST452	KM972292
YS93	NCL3-1	Polyagglutinating	No capsule	2012	Jiangsu Province	6	ST452	KM972293
YS94	NCL1-1	Polyagglutinating	20–30	2012	Jiangsu Province	6	ST402	KM972294
YS95	NCL2-1	Polyagglutinating	20–40	2012	Jiangsu Province	6	NT	KM972295
YS96	NCL2-1	Nonagglutinating	20–30	2012	Jiangsu Province	6	ST432	KM972296
YS97	NCL2-1	Nonagglutinating	20–30	2012	Jiangsu Province	6	ST432	KM972297
YS98	NCL2-1	Nonagglutinating	20–30	2012	Jiangsu Province	6	ST432	KM972298
YS99	NCL2-1	Nonagglutinating	20–30	2012	Jiangsu Province	6	ST432	KM972299
YS100	NCL2-1	Nonagglutinating	20–30	2012	Jiangsu Province	6	ST432	KM972222
YS101	NCL1-7	Polyagglutinating	No capsule	2012	Jiangsu Province	6	ST433	KM972223
YS102	NCL1-7	Polyagglutinating	No capsule	2012	Jiangsu Province	6	ST433	KM972224
YS103	NCL2-2	Polyagglutinating	No capsule	2012	Jiangsu Province	6	ST403	KM972225
YS104	NCL8-1	Polyagglutinating	No capsule	2012	Jiangsu Province	6	ST434	KM972226
YS107	NCL8-2	Polyagglutinating	20–40	2012	Beijing	6	ST436	KM972227
YS109	NCL8-1	Polyagglutinating	20–40	2012	Beijing	6	ST453	KM972228
YS110	NCL8-1	Polyagglutinating	20–30	2012	Beijing	6	ST453	KM972229
YS111	NCL8-1	Polyagglutinating	20–30	2012	Beijing	6	ST453	KM972230
YS113	NCL3-1	Polyagglutinating	35–55	2012	Beijing	6	ST437	KM972231
YS114	NCL1-2	Polyagglutinating	40–60	2012	Beijing	6	ST455	KM972232
YS117	NCL8-1	Polyagglutinating	No capsule	2012	Beijing	6	ST404	KM972233
YS121	NCL7-1	Polyagglutinating	20–40	2012	Beijing	6	ST407	KM972234
YS122	NCL8-1	Polyagglutinating	No capsule	2012	Beijing	6	ST408	KM972235
YS123	NCL8-1	Nonagglutinating	30–50	2012	Beijing	6	ST453	KM972236
YS125	NCL8-1	Nonagglutinating	30–50	2012	Beijing	6	ST453	KM972237
YS129	NCL1-2	Polyagglutinating	No capsule	2012	Beijing	6	ST439	KM972238
YS131	NCL8-1	Polyagglutinating	30–50	2012	Beijing	6	ST453	KM972239
YS132	NCL8-1	Nonagglutinating	20–30	2012	Beijing	6	NT	KM972240
YS133	NCL4	Polyagglutinating	30–50	2012	Beijing	7-3	ST409	KM972241

(Continued on following page)

TABLE 1 (Continued)

Isolate	*cps* locus type	Seroagglutination test result	Capsule thickness (nm)	Yr of isolation	Geographic location	MCG group[a]	MLST[b]	*cps* GenBank accession no.
YS140	NCL7-1	Polyagglutinating	20–40	2012	Beijing	6	ST442	KM972242
YS142	NCL1-2	Polyagglutinating	50–70	2012	Beijing	6	ST411	KM972243
YS145	NCL8-1	Polyagglutinating	15–25	2012	Beijing	6	ST443	KM972244
YS146	NCL8-3	Polyagglutinating	70–90	2012	Jiangsu Province	6	ST444	KM972245
YS147	NCL3-1	Polyagglutinating	No capsule	2012	Sichuan Province	6	ST462	KM972246
YS148	NCL2-3	Nonagglutinating	20–30	2012	Sichuan Province	7-2	ST463	KM972247
YS149	NCL1-1	Nonagglutinating	50–70	2012	Sichuan Province	6	ST464	KM972248
YS150	NCL3-1	Polyagglutinating	20–40	2012	Sichuan Province	6	ST465	KM972249
YS155	NCL2-1	Polyagglutinating	20–40	2012	Guizhou Province	6	ST446	KM972250
YS159	NCL8-1	Polyagglutinating	No capsule	2012	Guizhou Province	6	ST448	KM972251
YS160	NCL1-4	Nonagglutinating	No capsule	2012	Guizhou Province	6	ST467	KM972252
YS166	NCL3-1	Nonagglutinating	No capsule	2012	Beijing	6	ST469	KM972253
YS167	NCL1-5	Nonagglutinating	No capsule	2012	Beijing	6	ST470	KM972254
YS168	NCL1-5	Nonagglutinating	No capsule	2012	Beijing	6	ST470	KM972255
YS170	NCL3-1	Nonagglutinating	20–30	2012	Beijing	6	ST469	KM972256
YS171	NCL3-2	Polyagglutinating	20–30	2012	Beijing	6	ST472	KM972257
YS173	NCL8-1	Polyagglutinating	40–60	2012	Beijing	6	NT	KM972258

[a] N, nongroupable.
[b] NT, nontypeable.

nonserotypeable by both coagglutination and PCR testing (11, 12). Serum for the seroagglutination test was purchased from the Statens Serum Institut (Copenhagen, Denmark).

Species identification and molecular typing of nontypeable strains. We used 16S rRNA primers (20) to amplify a nearly complete 16S rRNA gene from the 78 isolates. The *recN* and *gdh* genes were also analyzed for taxonomic assignment, as was done previously (21–23). The API 20 Strep biochemical identification system (bioMérieux, Hazelwood, MO) was also used to identify the strains. Multilocus sequence typing (MLST) and minimum core genome sequence (MCG) typing were performed according to the methods described previously (19, 24).

Sequencing of *cps* loci. Thirty-six isolates were sequenced by Illumina sequencing: (i) 13 isolates were sequenced in a previous study BioProject accession numbers (PRJNA171455, PRJNA171456, PRJNA171458, PRJNA171460, PRJNA171461, PRJNA171464, PRJNA171466, PRJNA171467, PRJNA171472, PRJNA171473, PRJNA171480, PRJNA171481, and PRJNA171483) (25), and (ii) 23 additional bacterial strains were sequenced by Illumina sequencing in this study. Each *cps* locus sequence was extracted from the draft genome sequence, and the open reading frames (ORFs) were identified and annotated on the basis of the methods previously reported (11). The TMHMM (v2.0) analysis program (http://www.cbs.dtu.dk/services/TMHMM/) was used to identify putative *wzy* genes. The distribution of novel *wzy* genes in an additional 42 isolates was investigated by multiplex PCR (26) for NCL1 to NCL7 and a single PCR for NCL8 (upstream primer, 5′-AAAATTTTCACTTCACCTCGAC; downstream primer, 5′-AATCTTCCAATCAATGCTACGA; annealing temperature, 58°C; product size, 390 bp). The isolates harboring the same *wzy* gene were clustered into the same NCL. Finally, the *cps* clusters of 42 isolates were sequenced by directed PCR and primer walking on the basis of the known sequence of each NCL.

Bioinformatics analysis. The BLASTN and PSI-BLAST software programs (http://blast.ncbi.nlm.nih.gov/Blast.cgi) were used to search several databases, including GenBank (www.ncbi.nlm.nih.gov/GenBank), and the genome sequences of 85 strains sequenced previously (25). Loci were annotated with the Artemis program. The Clusters of Orthologous Groups (COG) and Pfam protein motif databases were used to search for conserved protein domains. *cps* genes were named according to the nomenclature for the *S. suis* serotype 2 *cps* locus (27). The genes from a locus are numbered by a letter from A to Z, in order. We defined the 5′ and 3′ sides of the *cps* clusters and clustered the proteins of the *cps* genes into HGs by the methods described in a previous study (9). The genes having a global match region at <50% of the amino acid sequence and with an identity of <50% were identified to be novel HGs compared to the HGs (HG1 to HG291) of the 35 reference serotype strains. Novel HGs were assigned numerically from HG292 onwards, continuing on from the previous HG assignment. Novel HGs present only in all isolates of a single NCL were identified as NCL-specific HGs. The Artemis comparison tool (ACT) (28) was used to visualize the data.

Phylogenetic analysis. The *cpsA* to *cpsD* sequences from 35 serotype reference strains and 78 nontypeable isolates were used to generate a phylogenetic tree. We used the Recombination Detection Program (RDP; v3.0) to remove the recombinational single nucleotide polymorphisms in the *cpsA* to *cpsD* sequences. An alignment of the *cpsA* to *cpsD* sequences was generated using the CLUSTAL W program (29). A phylogenetic tree was constructed using the neighbor-joining method, and a distance measure was obtained using the Tamura and Nei model implemented in the program MEGA (v5) (30).

Identification of capsule. The presence or absence of a capsule was confirmed using electron microscopy (31, 32). Briefly, bacteria were grown overnight in Todd-Hewitt broth medium, harvested by centrifugation, and washed once in phosphate-buffered saline. The cells were fixed for 2 h at room temperature in 0.1 M cacodylate buffer (pH 7) containing 5% glutaraldehyde and 0.15% ruthenium red; then, polycationic ferritin (1 mg/ml) was applied for 30 min at 20°C. The cells were then harvested using centrifugation and washed 3 times in 0.1 M cacodylate buffer. Then, the bacterial cells were immobilized in 4% agar, washed five times in cacodylate buffer, and postfixed with 2% osmium tetroxide for 2 h. The samples were dehydrated in a graded series of acetone concentrations (30%, 50%, 70%, 90%, 100%). Samples were then dehydrated in propylene oxide for 10 min and embedded in Spurr low-viscosity resin. Thin sections were cut and stained with uranyl acetate and lead citrate. The sections were examined with a transmission electron microscope (TH7700; Hitachi) at an accelerating voltage of 80 kV.

The capsule thickness between the inner edge and the external edge of the capsular layer was measured (33). Each value is based on 25 to 30 measurements per experiment. Each experiment was done twice independently.

Nucleotide sequence accession numbers. The *cps* sequences of the 78 isolates were deposited in the GenBank database under accession numbers KM972222 to KM972299.

RESULTS

Species identification and molecular typing of the nontypeable isolates. All 78 nontypeable isolates were unambiguously identified to be *S. suis* by biochemical tests and comparative sequence analyses of the 16S rRNA, *recN*, and *gdh* genes (data not shown).

We further typed the 78 isolates by MLST, and 56 sequence types (STs) were found. The STs of four isolates could not be obtained due to PCR failure of one of the seven housekeeping genes (Table 1) (19). Using genome sequences, they could also be assigned to the MCG groups previously defined (19, 25). The majority of the isolates were from the most ancestral MCG group, group 6 (84.6%, 66/78), while a small proportion of the isolates belonged to group 7 (14.1%, 11/78 isolates). The MCG of one isolate was nongroupable (Table 1).

Molecular grouping of the 78 isolates on the basis of the *wzy* gene. The *wzy* gene has been used as a marker for molecular serotyping of *S. suis* (11, 12, 26, 34). When the 78 *wzy* sequences were compared with reference *wzy* sequences, they were clustered away from the reference sequences as 8 novel clusters. On the basis of these eight novel *wzy* genes, the *cps* gene clusters from these nontypeable isolates could be classified into eight different NCLs (Table 1). The number of isolates in each NCL varied: NCL1 had 25 isolates, NCL2 had 11 isolates, NCL3 had 12 isolates, NCL4 had 6 isolates, NCL5 had 2 isolates, NCL6 had 1 isolate, NCL7 had 4 isolates, and NCL8 had 17 isolates.

General features of the NCLs. Previous studies showed that not all *S. suis cps* gene clusters were located at the same chromosomal location, and 5 insertion sites were found (9). We found that all *cps* loci in these nontypeable strains were located between the *orfZ-orfX* region and the *glf* (UDP-galactopyranose mutase) gene, which was previously found to be the *cps* gene cluster insertion site for reference serotypes 9, 13, 21, 24, 29, 31, and 33 and was defined as location pattern I-b.

The sizes of the NCLs ranged from 15.3 kb to 32.8 kb, and the G+C content of the NCLs varied from 33.4 to 36.8%. The 5′ region of all eight NCLs was conserved, whereas the 3′ region was variable, with the 3′ regions of NCL1, NCL7, and NCL8 sharing high similarity and the 3′ region of NCL4 being nearly identical to that of NCL5 but differing from the 3′ regions of NCL1, NCL7, and NCL8. The central regions of the eight NCLs were highly variable (Fig. 1F). The polysaccharide polymerase (*wzy*) and flippase (*wzx*) genes were always present downstream together with various sets of genes coding for glycosyltransferase, acetyltransferase, and modifying enzymes. Sialic acid synthesis genes were not found in any of the NCLs.

The *cpsA*, *cpsB*, *cpsC*, and *cpsD* genes were present and located at the 5′ side of the NCLs. The nucleotide sequences of *cpsA* to *cpsD* were conserved across both NCLs and known reference *cps* loci. In the phylogenetic tree based on the nucleotide sequence alignment of the region from *cpsA* to *cpsD*, all our nontypeable strains were clustered together with reference strains with a *cps* locus of pattern I-a (reference serotypes 1, 1/2, 2, 3, 4, 5, 7, 8, 10, 11, 12, 14, 15, 17, 18, 19, 23, 25, 28, and 30) or pattern I-b (reference serotypes 9, 13, 21, 24, 29, and 31) and were distant from the strains with a *cps* locus of pattern I-a (reference serotypes 6 and 16), pattern I-b (serotype 33), pattern II (serotype 27), pattern III (serotypes 20, 22, and 26), and pattern IV (serotypes 32 and 34) (see Fig. S1 in the supplemental material).

Comparison of HG contents of NCLs with those of reference *cps* gene clusters. A previous study classified the *S. suis cps* genes in the reference serotypes into HGs, with 291 HGs being named (9). Using the same criteria used previously, the predicted coding sequences in the eight NCLs could be divided into 96 HGs (see Table S1 in the supplemental material). Twenty-six of these 96 HGs were present in the *cps* loci of the serotype reference strains, while 70 were novel HGs not found in the serotype reference strains and were named HG292 to HG361. Fifty-five novel HGs were NCL specific. Each NCL contained 4 to 10 NCL-specific HGs (see Table S1 in the supplemental material). Eight HGs (HG312, HG313, HG314, HG315, HG329, HG332, HG354, and HG355) were variably present within an NCL. Seven HGs (HG292, HG293, HG294, HG295, HG316, HG330, and HG331) were present in at least two NCLs. Some of the specific enzymes are described below.

(i) Initial sugar transferases. The initial glycosyl phosphotransferase is responsible for linkage of an activated glycosyl phosphate to the lipid carrier (35). Similar to the serotype reference strains, the initial sugar transferase genes were located in the 5′ region in all NCLs and were classified into 4 HGs: HG6 (NCL6), HG8 (NCL4 and NCL8), HG21 (NCL1 and NCL5), and HG295 (NCL2, NCL3, and NCL7) (see Table S1 in the supplemental material).

(ii) Glycosyltransferases. The glycosyltransferase catalyzes the attachment of sugars to an aglycone in CPS synthesis. Except for the initial glycosyl phosphotransferase, the other glycosyltransferases in all strains tested fell into 31 HGs, and only three of the glycosyltransferases (HG45, HG81, and HG99) were present in serotype reference strains.

(iii) Other transferases. Acetyltransferase plays an important role in CPS structure determination (36). Acetyltransferases in all tested strains fell into six HGs, only one of which (HG104) existed in a serotype reference strain. The others were NCL-specific novel HGs. Aminotransferase is identified to be an enzyme which can transfer amino groups to sugars (37). Two aminotransferases (HG22 and HG41) were present in the NCLs. Cytidylyltransferases (HG66 and HG347), nucleotidyltransferases (HG313 and HG352), a hexosyltransferase (HG357), and a LicD-family phosphotransferase (HG334) were also present in the NCLs.

(iv) Wzy polymerase and Wzx flippase. The Wzy polymerase was quite different in each of the eight NCLs and belonged to NCL-specific HGs. The sequences of *wzy* were highly conserved within the same group.

Eight Wzx flippases were present in NCLs. Except for NCL4, the Wzx flippases of the other seven NCLs also belonged to NCL-specific HGs. The NCL4 Wzx flippase was homologous to the Wzx flippases of reference serotypes 8, 9, and 33 (HG43).

Structural variation within NCLs. We found genetic structural variations in five of the eight NCLs with the insertion, deletion, or truncation of genes. No genetic heterogeneity was found within NCL4, NCL5, or NCL6, and these NCLs contained few isolates.

(i) NCL1. Seven subtypes were found in NCL1 (Fig. 1A; see also Table S1 in the supplemental material): NCL1-1 (7 strains), NCL1-2 (7 strains), NCL1-3 (3 strains), NCL1-4 (2 strains), NCL1-5 (2 strains), NCL1-6 (2 strains), and NCL1-7 (2 strains). The subtypes varied due to the variable presence of eight HGs (HG55, HG293, HG294, HG312, HG313, HG314, HG315, and HG329) and transposase genes. Subtype NCL1-7 differed from NCL1-1 by the insertion of a transposase gene. Simi-

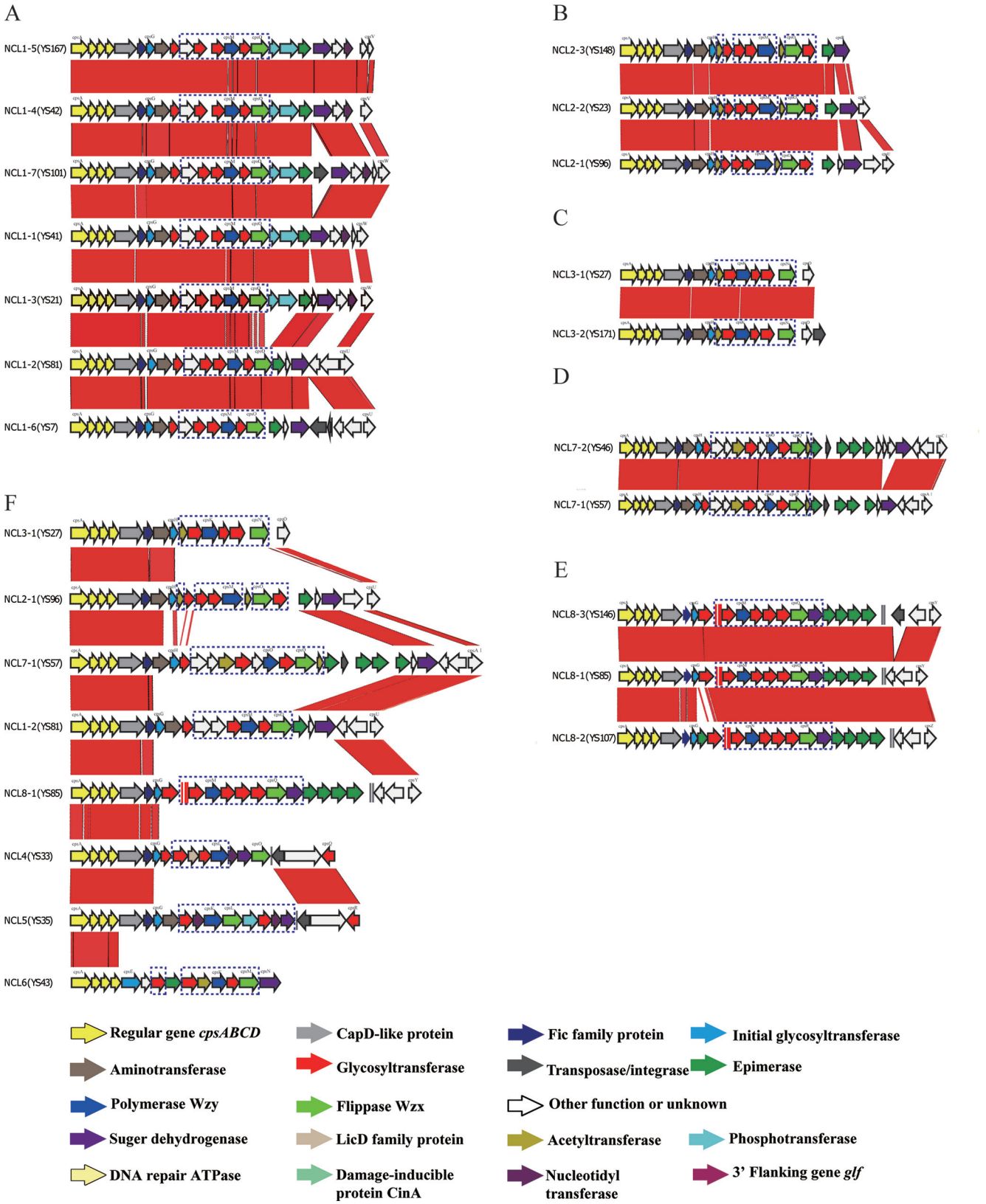

FIG 1 Comparison of the *cps* loci within NCL1 (A), NCL2 (B), NCL3 (C), NCL7 (D), NCL8 (E), and 8 NCLs (F). Each colored arrow represents the gene whose predicted function is identified at the bottom. The names of the first and last genes, initial transferase genes, and the *wzx* and *wzy* genes of each NCL are appended to the corresponding arrows. NCL-specific genes are indicated by dotted blue lines. The red bars in each panel indicate regions conserved among strains. The *glf* gene is located on the 3′ side of each locus and is not marked.

larly, differences between NCL1-4 and NCL1-5 and between NCL1-2 and NCL1-6 were also due to transposase gene insertions. NCL1-7 differed from NCL1-4 by the insertion of a transposase gene and the deletion of HG329. NCL1-1 differed from NCL1-3 by the insertion of HG329 and the deletion of HG55. NCL1-2 differed from NCL1-3 by the insertion of HG312, HG313, HG314, and HG315 and the deletion of HG293 and HG294.

(ii) NCL2. Three types of genetic organizations were found in NCL2 (Fig. 1B; Table 1): NCL2-1 (7 strains), NCL2-2 (3 strains), and NCL2-3 (1 strain). In comparison with NCL2-1, two genes (HG55 and HG332) were deleted in NCL2-2, whereas three genes (HG55, HG292, and HG332) were deleted in NCL2-3.

(iii) NCL3. Two types of genetic organizations were found in NCL3 (Fig. 1C; Table 1): NCL3-1 (11 strains) differed from NCL3-2 (1 strain) by a transposase gene inserted in the 3′ region in NCL3-2.

(iv) NCL7. Two types of genetic organizations were found in NCL7 (Fig. 1D, Table 1): NCL7-1 (3 strains) differed from NCL7-2 (1 strain) by two genes (HG354 and HG355) which were inserted in the 3′ region in NCL7-2.

(v) NCL8. Three types of genetic organizations were found in NCL8 (Fig. 1E, Table 1): NCL8-1 (15 strains), NCL8-2 (1 strain), and NCL8-3 (1 strain). Compared to the sequence of NCL8-1, NCL8-2 had an extra gene (HG23) inserted in the 5′ region. A truncation by the insertion of a transposase gene was found in HG293 (*cpsW*) of NCL8-3.

Capsule analysis by transmission electron microscopy. The thickness of the capsule of the 78 isolates was measured by transmission electron microscopy (Table 1; representative strains are shown in Fig. 2). Capsule analysis was performed twice for each strain, and the capsule thickness was consistent between the experiments. Strain SC84 (serotype 2) was used as a control and was well encapsulated, and its capsule had a thickness of from 110 nm to 130 nm. Fifty-three nontypeable strains were most likely encapsulated with varied capsule thicknesses (Table 1). The capsule thickness also showed wide variation between strains within an NCL or NCL subtypes. The remaining 25 nontypeable strains are likely to be nonencapsulated (Table 1). The nonencapsulated strains were distributed among the different NCLs and subtypes.

DISCUSSION

In this study, we analyzed the *cps* loci of 78 nontypeable strains isolated from healthy pigs and found eight NCLs with 70 novel HGs. The isolates that harbored identical novel *cps* gene clusters were from diverse geographic locations and belonged to different MLST types, suggesting that these strains have been widely circulating in the pig population. They shared highly conserved *cpsA*, *cpsB*, *cpsC*, and *cpsD* genes with known pattern I-a and pattern I-b serotype reference strains, indicating that they probably evolved from a common ancestor. The possession of *wzy* and *wzx* genes in NCLs strongly suggests that their CPSs were synthesized by the Wzx/Wzy-dependent pathway. The sequences of NCL-specific *wzy* genes were quite variable in different types of *cps* loci but were highly conserved within the same group. Consistent with previous reports, *wzy* is an ideal target with which to discriminate the *cps* loci in nontypeable *S. suis* strains.

By comparison with known *cps* loci of serotype reference strains, it was found that genes responsible for the synthesis and polymerization of the repeat unit were located in the central region of the gene cluster and were highly variable in the NCLs. The 70 novel HGs are likely to have been acquired by lateral gene transfer from other species. The source of these novel genes is unclear, but they are likely to have originated from the microbiota in the nasopharynx, which provides a large gene pool for *S. suis cps* loci. The exchange of these genes may be facilitated by the transposase-like regions which were found in the NCLs. Further, as *cps* gene reservoirs, the NCLs may play an important role in generating the *cps* gene cluster variation in *S. suis* CPS by intraspecies gene transfer. The presence of many different *cps* loci in *S. suis* suggests a continuous and ongoing evolution of *cps* gene clusters in this species.

Among the 55 NCL-specific novel HGs, 25 genes encoded glycosyltransferases and 9 genes encoded additional transferases. The presence of these unique transferases implies that the oligosaccharide structure repeat units that they transfer are unique. Most of the NCL-specific novel HGs showed weak similarity to the products of genes of other species. However, NCL-specific novel HGs of NCL8 (from HG305 to HG311) showed high similarity to *S. pneumoniae* serotype 12A *cps* genes (from SPC12A_0012 to SPC12A_0018). These genes are also conserved in *S. pneumoniae* serotypes 12B, 12F, 44, and 46. Homologs of the L-Fuc*p*NAc pathway and D-Mna*p*NAcA pathway genes present in *S. pneumoniae* serotypes 12A and 12F were also found in NCL8. Thus, the oligosaccharide structure repeat unit in NCL8 may be similar to the oligosaccharide structure repeat units of *S. pneumoniae* serotypes 12, 44, and 46 and different from those of *S. suis* serotypes 2 and 14 (38, 39). A similar observation was also made for NCL6. Homologs of some *S. pneumoniae* serotype 9N *cps* genes (10) were found in NCL6 (HG357, HG359, HG360, and HG361).

It is interesting to note that encapsulated strains predominantly distributed in NCL1-1, NCL1-2, NCL2-1, NCL3, NCL5, NCL7-1, and NCL8 in this study. Pan et al. also reported on the isolation of a novel *S. suis* serotype from pigs with meningitis (34). The *cps* locus of a novel variant serotype was similar to NCL1 on the basis of sequence data, whereas they shared *wzx* (HG301) and six other novel HGs (HG292, HG300, HG312, HG313, HG314, and HG315). We also found strains that harbor NCL1, NCL3, and NCL7 from the lungs of diseased pigs (our unpublished data). CPS plays important roles in *S. suis* colonization of the host, resistance to phagocytosis, and persistence in the blood (40–42). Therefore, further characterization of strains isolated from diseased pigs is needed to determine the importance of these strains harboring NCL1, NCL2, NCL3, NCL5, NCL7, and NCL8 as disease-causing serotypes.

A total of 25 isolates were nonencapsulated. These strains were mainly distributed in two NCLs (NCL4 and NCL6) and seven subtypes (NCL1-4 to NCL1-7, NCL3-1, NCL7-2, and NCL8-1). Since these strains, as well as capsulated *S. suis*, can colonize pig nasopharynges, it is possible that some of these strains may produce a thin capsule that was present at levels below the detection limit of the method that we used or the capsule is produced *in vivo*. In this study, we used polycationic ferritin as a substitute for homologous antiserum to stabilize the capsule during the dehydration steps (33, 43, 44). It is possible that polycationic ferritin incompletely stabilized the capsular material, leading to the collapse of the thin capsule during the dehydration process. It is also possible that some of these strains were defective in CPS production because of a defective *cps* gene cluster. HG34 in strains YS160, YS167, and YS168 was truncated by a nonsense mutation. The glycosyltransferase genes, HG297 in strain YS160 and HG300 in

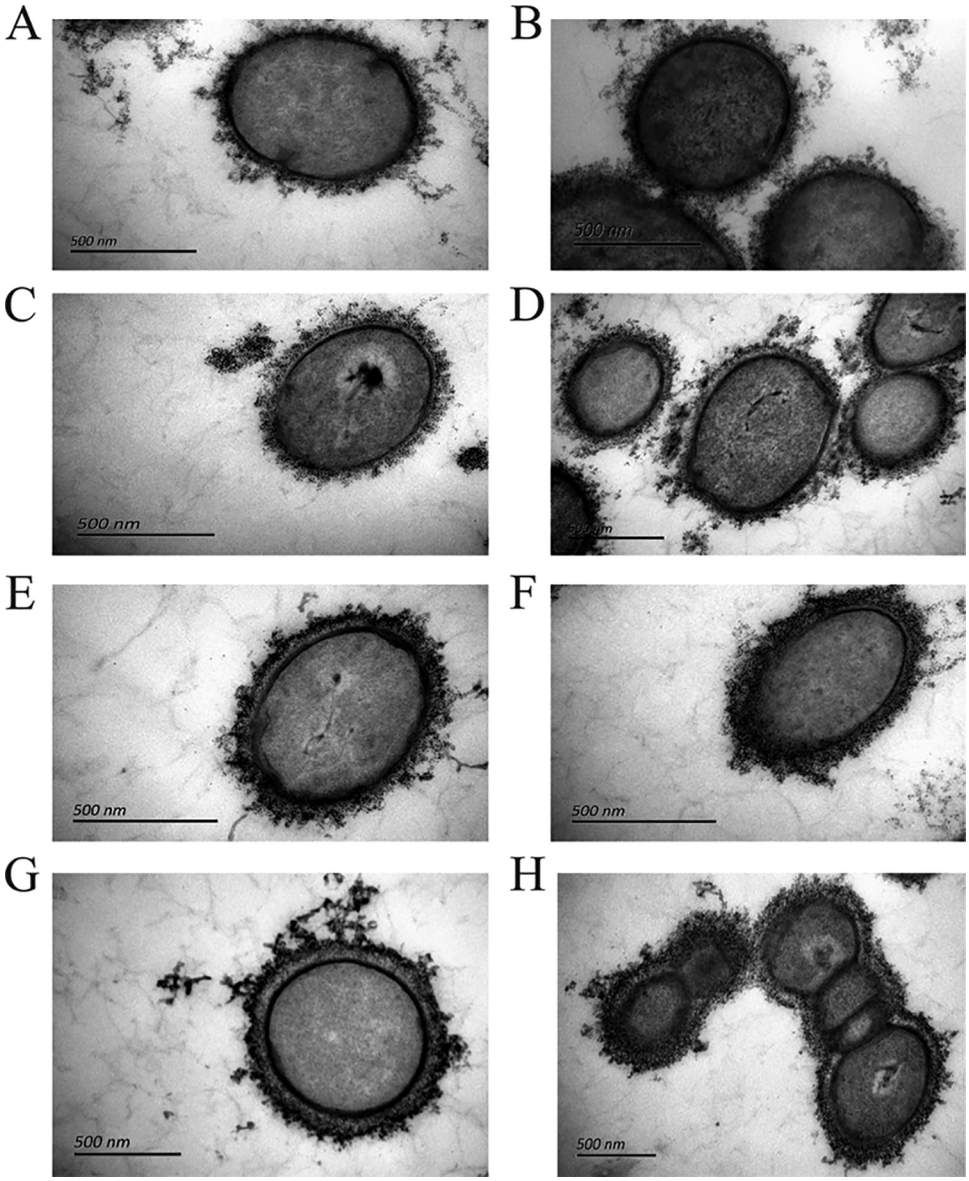

FIG 2 Visualization of the *S. suis* capsule of representative strains using transmission electron microscopy following ferritin stabilization. (A) YS74 (NCL3-1); (B) YS121 (NCL7-1); (C) YS35 (NCL5); (D) YS72 (NCL2-2); (E) YS133 (NCL4); (F) YS149 (NCL1-1); (G) YS146 (NCL8-3); (H) *S. suis* serotype 2 control strain SC84.

YS103, were disrupted by a single base deletion, resulting in a frameshift mutation. The gene HG292 in YS167 and YS168 was disrupted by the insertion of a transposase gene. These truncation mutations, along with the other nonsynonymous changes, may account for the defective *cps* gene cluster. In addition, it is known that certain genes outside the *cps* locus are involved in the modification or transcriptional regulation of capsular polysaccharides in other species (45), and thus, the lack of production of a capsule may not be due to a defect in the *cps* gene cluster.

In conclusion, we determined the genetic characteristics of the *cps* loci from nontypeable *S. suis* isolates, and the novel genes identified facilitated their discrimination. Our data also contribute to the understanding of the genetic diversity of the *cps* loci in nontypeable *S. suis* isolates and provide insight into the evolution of capsular genes in *S. suis*.

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