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The Minor Pilin Subunit Sgp2 Is Necessary for Assembly of the Pilus Encoded by the *srtG* Cluster of *Streptococcus suis*▽†

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Gram-positive pili are composed of covalently bound pilin subunits whose assembly is mediated via a pilus-specific sortase(s). Major subunits constitute the pilus backbone and are therefore essential for pilus formation. Minor subunits are also incorporated into the pilus, but they are considered to be dispensable for backbone formation. The *srtG* cluster is one of the putative pilus gene clusters identified in the major swine pathogen *Streptococcus suis*. It consists of one sortase gene (*srtG*) and two putative pilin subunit genes (*sgp1* and *sgp2*). In this study, by constructing mutants for each of the genes in the cluster and by both immunoblotting and immunogold electron microscopic analysis with antibodies against Sgp1 and Sgp2, we found that the *srtG* cluster mediates the expression of pilus-like structures in *S. suis* strain 89/1591. In this pilus, Sgp1 forms the backbone, whereas Sgp2 is incorporated as the minor subunit. In accordance with the current model of pilus assembly by Gram-positive organisms, the major subunit Sgp1 was indispensable for backbone formation and the cognate sortase SrtG mediated the polymerization of both subunits. However, unlike other well-characterized Gram-positive bacterial pili, the minor subunit Sgp2 was required for polymerization of the major subunit Sgp1. Because Sgp2 homologues are encoded in several other Gram-positive bacterial pilus gene clusters, in some types of pili, minor pilin subunits may contribute to backbone formation by a novel mechanism.

Pilus-like structures on the surface of Gram-positive bacteria were first observed in *Corynebacterium renale* by electron microscopy (50), and recently, these surface appendages have been characterized genetically and biochemically in many additional Gram-positive bacterial pathogens (18, 33, 44, 46). Gram-positive bacterial pili are anchored to the cell wall peptidoglycan and consist of covalently cross-linked subunit proteins (18, 33, 44, 46). Polymerized monomers of a single major pilin subunit form the pilus backbone, to which one or more minor (or ancillary) pilin subunits are attached (18, 33, 44, 46). Both the major and minor subunits contain C-terminal cell wall sorting signals (CWSSs), composed of a pentapeptide motif represented by LPXTG (where X is any amino acid), a C-terminal hydrophobic domain, and a charged tail (32). The subunits are assembled via CWSSs by the action of pilus-specific class C sortases (8).

Major pilin subunits have been shown to be indispensable for pilus formation. In contrast, early studies showed that minor subunits are not essential for pilus synthesis and assembly (10, 39, 47). It has only recently been reported that minor pilin subunits of SpaA- and SpaH-type pili of *Corynebacterium diphtheriae* and Spy0130 (FctB) of a *Streptococcus pyogenes* serotype M1 strain participate in the termination of pilus polymerization as well as in anchoring the polymer to the cell wall peptidoglycan (17, 35). However, in many Gram-positive bacterial pili, the roles of minor pilin subunits in pilus formation remain to be fully elucidated.

Streptococcus suis is a Gram-positive coccus responsible for a wide range of diseases in pigs, including meningitis, septicemia, endocarditis, and sudden death (14, 37). This bacterium can also affect humans in close contact with diseased pigs or swine products (1, 7, 43, 49, 51). Four putative pilus gene clusters, named the *srtBCD*, *srtE*, *srtF*, and *srtG* clusters, have so far been identified in *S. suis* (9, 40). The *srtG* cluster, identified in strain 89/1591, consists of one putative sortase gene, *srtG*, and two putative pilin subunit genes, *sgp1* and *sgp2* (Fig. 1A). The genetic organization of the *srtG* cluster was similar to that of the fibronectin-binding, collagen-binding, T-antigen 1 (FCT-1) region of *S. pyogenes* (21). Moreover, the amino acid sequences of SrtG, Sgp1, and Sgp2 showed 35 to 56% identity with those of the proteins encoded by the corresponding genes for the FCT-1 region (40). However, despite the *in silico* predictions, it is unknown whether this cluster is associated with the expression of pilus fibers in *S. suis*.

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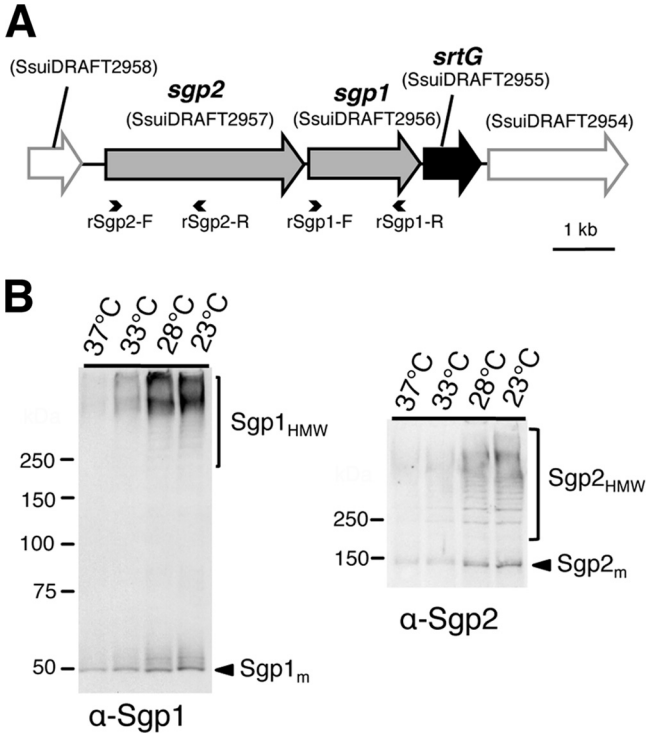

FIG. 1. Surface expression of Sgp1 and Sgp2 in wild-type strain 89/1591. (A) Genetic organization of the *srtG* pilus cluster. This schema is constructed on the basis of the draft genome sequence of *S. suis* 89/1591, updated in February 2009 (GenBank accession no. AAFA03000013). Gray arrows, genes encoding CWSS-containing proteins (*sgp1* and *sgp2*); black arrow, sortase-encoding gene (*srtG*); open arrows outlined in gray, genes flanking the cluster; small arrowheads, annealing positions of the primers used to generate His-tagged recombinant Sgp1 and Sgp2. (B) Immunoblots with anti-Sgp1 (α-Sgp1; left panel) and anti-Sgp2 (α-Sgp2; right panel) rabbit pAbs using bacterial cells at different growth temperatures (37°C, 33°C, 28°C, or 23°C). Bacteria were grown in THB until the optical density at 600 nm reached 0.35, which corresponds to the mid-log phase. Each lane contains cell wall fractions extracted from cells in 750-μl cultures with an optical density at 600 nm of 0.35. The calculated molecular weights of $Sgp1_m$ and $Sgp2_m$ are 52,045 and 104,403, respectively. Numbers to the left of the gels are molecular weights (in thousands).

In this study, we generated polyclonal antibodies (pAbs) against the two putative pilin subunits (Sgp1 and Sgp2) and identified reactive pilus-like appendages on the surface of *S. suis* 89/1591 by both immunoblotting and immunogold electron microscopy (IEM). By constructing a series of isogenic mutant strains, we demonstrated that the *srtG* cluster mediates pilus formation in *S. suis* 89/1591 and that the minor subunit Sgp2 is necessary for polymerization of the major subunit Sgp1.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *S. suis* strains were grown in Todd-Hewitt (Becton Dickinson, Sparks, MD) broth (THB) or agar (THA) at 37°C in a 5% CO_2 atmosphere, unless otherwise specified. *Escherichia coli* strains were cultured in Luria-Bertani (Becton Dickinson) broth or agar at 37°C. When required, the following antibiotics were added to the medium at the indicated concentrations: for *E. coli*, ampicillin, 50 μg/ml; chloramphenicol, 25 μg/ml; and spectinomycin, 50 μg/ml; for *S. suis*, spectinomycin, 100 μg/ml. When necessary, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside was added to the plates at 100 μg/ml.

DNA techniques. Restriction enzymes and DNA-modifying enzymes were purchased from Takara Bio (Otsu, Japan) and used according to the manufacturer's recommendations. Purification of plasmid DNA from *E. coli* and transformation of *E. coli* were performed using standard procedures (31). Genomic DNA of *S. suis* was extracted by the method described previously (25), and *S. suis* was transformed by electroporation, as described previously (41). Ex *Taq* polymerase (Takara Bio) and iProof HF master mix (Bio-Rad Laboratories, Hercules, CA) were used for PCR amplification. Primers used in this study are listed in Table S1 in the supplemental material. Sequencing was carried out with a BigDye Terminator (version 3.1) cycle sequencing kit (Applied Biosystems, Foster City, CA) and analyzed on a 3100 genetic analyzer or 3130xl genetic analyzer (Applied Biosystems).

Generation of *S. suis* mutants. For the construction of precise in-frame deletions in *srtG*, *sgp1*, and *sgp2*, upstream and downstream regions of these genes were amplified by PCR and fused by overlap-extension PCR (48). For insertional inactivation of *cps2B*, an internal region of the gene was amplified. The resulting PCR products were cloned into pCR2.1 (Invitrogen, San Diego, CA), excised with EcoRI, and recloned into the EcoRI site of temperature-sensitive *S. suis-E. coli* shuttle vector pSET4s (42). The resulting plasmids were introduced into *S. suis* 89/1591 by electroporation. The single-crossover mutant (CPS2B) and double-crossover mutants (Δ*srtG*, Δ*sgp1*, and Δ*sgp2*) were generated according to the procedures described previously (42) with slight modifications. Briefly, the single-crossover mutants were obtained by culturing the cells on THA with spectinomycin at 37°C, and the double-crossover mutants were generated by repeated passaging at 28°C on THA without the antibiotic. The deletion or inactivation of target genes was confirmed by PCR and sequence analyses.

Complementation of deletion mutants. The pMX1 vector was used for the generation of recombinant plasmids for complementation analysis (Table 1). This vector is a derivative of the *S. suis-E. coli* shuttle cloning vector pSET2 (41) and possesses the *S. suis malX* promoter for transgene expression in *S. suis*. The entire *srtG*, *sgp1*, and *sgp2* genes were amplified from genomic DNA of *S. suis* 89/1591 and cloned into pMX1 via EcoRI and BamHI sites (*srtG* and *sgp1*) or the NcoI site (*sgp2*), generating complementation vectors pSrtG, pSgp1, and pSgp2, respectively. These plasmids were introduced into *E. coli* MC1061 for verification of the sequences and then into the respective deletion mutants derived from *S. suis* 89/1591 to construct *srtG*-, *sgp1*-, and *sgp2*-complemented mutants.

Preparation of His-tagged recombinant proteins and polyclonal antibodies. Internal fragments of *sgp1* and *sgp2* were amplified with primer sets rSgp1-F/rSgp1-R and rSgp2-F/rSgp2-R (Fig. 1A), respectively, and cloned into pCR2.1. After excision with NdeI and BamHI, the fragments were ligated into the pIVEX 2.4d vector (Roche Applied Science, Basel, Switzerland). The resulting products were introduced into *E. coli* TOP10 for verification of the sequences and *E. coli* BL21(DE3)/pDIA17 (28) for protein expression. Recombinant protein synthesis was induced with isopropyl-β-D-thiogalactopyranoside, as previously described (28). Recombinant proteins were purified by affinity chromatography on nickel-nitrilotriacetic acid columns (Protino protein purification system; Macherey-Nagel, Düren, Germany), according to the manufacturer's instructions. Protein purity was checked by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), and protein concentrations were determined accurately using a bicinchoninic acid protein assay kit (Pierce Biotechnology, Rockford, IL).

Purified proteins were used for polyclonal antibody production in rabbits at Iwaki Corporation (Tokyo, Japan) and in mice at Operon Biotechnologies (Tokyo, Japan). As necessary, the resulting rabbit antibodies were purified with a HiTrap Protein G HP column (GE Healthcare, Piscataway, NJ) and a Microcon YM-10 centrifugal filter device (Millipore, Bedford, MA), according to the manufacturers' recommendations. Purification of mouse antiserum was performed by Operon Biotechnologies.

Preparation of bacterial cell wall, protoplast, and culture supernatant fractions. *S. suis* cultures were harvested, washed once with TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), suspended in spheroplasting buffer (20 mM Tris-HCl [pH 6.8], 10 mM $MgCl_2$, 26% raffinose, 500 units/ml mutanolysin, 1 tablet/10 ml complete mini-EDTA-free protease inhibitor cocktail [Roche]) (20), and incubated for 1 h at 37°C to digest peptidoglycan with mutanolysin. After centrifugation at 13,000 × *g* for 5 min at 4°C, the supernatants including digested peptidoglycan and released cell wall-associated proteins were separated from protoplasts and collected as cell wall fractions. The protoplast pellets were washed once with protoplast buffer (20 mM Tris-HCl [pH 6.8], 10 mM $MgCl_2$, 26% raffinose) and suspended in phosphate-buffered saline (PBS; pH 7.4) containing 2% Triton X-100. Culture supernatants were filtered through a 0.22-μm-pore-size filter, precipitated by trichloroacetic acid, washed twice with acetone, and dried.

TABLE 1. Plasmids and bacterial strains used in this study

Plasmid or strain	Relevant properties	Source or reference
Plasmids		
pCR2.1	Ap^r Km^r, pUC *ori*, f1 *ori*, *lacZ*ΔM15	Invitrogen
pDIA17	Cm^r, p15A *ori*, Tet promoter, *lacI*	22
pIVEX2.4d	Ap^r, pUC *ori*, T7 promoter, His tag-coding sequence	Roche
pSET4s	Spc^r, pUC *ori*, thermosensitive pG+host3 *ori*, *lacZ*ΔM15	42
pMX1	Spc^r, pSSU1 *ori*, *malX* promoter of *S. suis*, derivative of pSET2	This study
pSrtG	pMX1 carrying intact *srtG* gene	This study
pSgp1	pMX1 carrying intact *sgp1* gene	This study
pSgp2	pMX1 carrying intact *sgp2* gene	This study
S. suis strains		
89/1591	Wild-type strain isolated from a pig with septicemia and meningitis	30
Δ*srtG*	89/1591 derivative strain carrying an in-frame deletion in *srtG*	This study
Δ*sgp1*	89/1591 derivative strain carrying an in-frame deletion in *sgp1*	This study
Δ*sgp2*	89/1591 derivative strain carrying an in-frame deletion in *sgp2*	This study
CPS2B	89/1591 derivative strain carrying an insertionally inactivated *cps2B* (*cps2B*::pSET4s)	This study
E. coli strains		
TOP10	Host for pCR2.1 and pSET4s derivatives	Invitrogen
BL21(DE3)/pDIA17	Host for pIVEX2.4d derivatives	28
MC1061	Host for pMX1 derivatives	6

Coimmunoprecipitation. The cell wall and protoplast fractions extracted from 15-ml cultures grown overnight at 28°C were incubated with 1 μg purified anti-Sgp1 or anti-Sgp2 rabbit pAb at room temperature (RT) for 1 h with end-over-end rotation. For coimmunoprecipitation, 25 μl protein G Dynabeads (Invitrogen) was added to the mixtures, and the mixtures were incubated at RT for 1 h with end-over-end rotation. Immunoprecipitated complexes bound to protein G beads were then pulled down and washed four times with PBS containing 2% Triton X-100 using a side-pull magnetic isolation apparatus (Invitrogen). Isolated beads were used for immunoblot analysis as described below.

Immunoblot analysis. Prepared fractions or isolated protein G beads were boiled in SDS-PAGE sample buffer (50 mM Tris-HCl [pH 6.8], 2% SDS, 0.5% bromophenol blue, 10% glycerol, 60 μl/ml β-mercaptoethanol) for 10 min before gel electrophoresis. The samples were separated by 10% precast polyacrylamide gels (Atto Corp., Tokyo, Japan) and transferred to nitrocellulose (Whatman, Kent, United Kingdom) or polyvinylidene fluoride (Bio-Rad) membranes. The membranes were blocked with blocking solution (Western blocking reagent [Roche] diluted 1:10 in maleic acid buffer [Roche]) for 2 h at RT and were subsequently incubated at RT with anti-Sgp1 or anti-Sgp2 pAb diluted 1:1,000 in blocking solution (for anti-Sgp1 pAb) or Can Get signal immunoreaction enhancer solution 1 (for anti-Sgp2 pAb) (Toyobo Corp., Osaka, Japan) for 1 h. The membranes were then washed three times with washing buffer (diluted 1:10 in distilled water; Roche) and incubated at RT with alkaline phosphatase-conjugated anti-rabbit or anti-mouse IgG (MP Biomedicals, Irvine, CA) diluted 1:5,000 in washing buffer (for Sgp1 detection) or Can Get signal immunoreaction enhancer solution 2 (for Sgp2 detection) for 1 h. After three washes with washing buffer, Sgp1 or Sgp2 was detected with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate substrates (Roche) or CDP-Star ready-to-use chemiluminescent substrates (Roche). Densitometry analysis of immunoblots developed with chemiluminescent substrates was performed using a VersaDoc imaging system and Quantity One software (Bio-Rad). For detection of differences, densities were compared by Student's *t* test.

Partial purification of *srtG* pili. *S. suis* strains were grown for 12 h at 28°C in 200 ml THB, harvested, washed once in PBS, suspended in 1 ml modified spheroplasting buffer (containing 20% sucrose instead of 26% raffinose), and incubated at 37°C for 7 h with gentle shaking to digest peptidoglycan with mutanolysin. After incubation, supernatants containing released pilus material were loaded onto sucrose gradients (25 to 55% in 20 mM Tris-HCl [pH 6.8], 10 mM $MgCl_2$) and centrifuged at 25,000 × *g* for 20 h by a Beckman L-60 ultracentrifuge (Beckman-Coulter, Fullerton, CA) with an SW41Ti rotor at 4°C. Collected gradient fractions were tested for the presence of pilus subunits by immunoblotting. The fractions containing subunit polymers were pooled, treated with benzonase nuclease (Novagen, Madison, WI) to remove DNA and RNA impurities, and dialyzed against 20 mM Tris-HCl (pH 6.8)–10 mM $MgCl_2$ to remove sucrose.

IEM. *S. suis* strains were grown for 12 h at 28°C in 5 ml THB, harvested by centrifugation, washed once with PBS, and suspended in approximately 5 ml PBS containing 2% paraformaldehyde (PFA). After fixation for 5 min, the bacterial suspensions were placed on collodion-coated copper grids (Nisshin EM, Tokyo, Japan) for 10 min and allowed to dry. For mounting partially purified pili, 10-μl samples were placed on grids for 5 min, fixed with PBS containing 2% PFA for 5 min, and allowed to dry. Grids were subsequently blocked with 10% normal horse serum in dilution buffer (PBS containing 1% bovine serum albumin and 1% Tween 20, pH 7.4) for 30 min at RT. Thereafter, the grids were soaked in anti-Sgp1 mouse pAb diluted 1:20 in dilution buffer for 1 h and washed three times with dilution buffer. The grids were then soaked in 10-nm gold-conjugated goat anti-mouse IgG (British BioCell International, Cardiff, United Kingdom) diluted 1:30 in dilution buffer, incubated for 1 h at RT, and washed five times with dilution buffer. For double-labeling experiments, the same procedure was further applied using anti-Sgp2 rabbit pAb and 20-nm gold-conjugated goat anti-rabbit IgG. Finally, samples were fixed in PBS containing 2% PFA for 10 min at RT, washed eight times with distilled water, and allowed to dry. The grids were observed with an H7650 electron microscope (Hitachi Ltd., Tokyo, Japan) at an accelerating voltage of 80 kV.

RESULTS

Sgp1 and Sgp2 are expressed on the bacterial cell surface and polymerized by cognate SrtG. Polymerized pilin subunits of Gram-positive bacteria cannot be dissociated by boiling in SDS-PAGE sample buffer because of covalent subunit-subunit linkages and thus can be detected as a high-molecular-weight (HMW) ladder by SDS-PAGE immunoblotting (33, 46, 47). To investigate whether HMW polymers of putative pilin subunits Sgp1 and Sgp2 ($Sgp1_{HMW}$ and $Sgp2_{HMW}$, respectively) are expressed on the cell surface, we extracted the cell wall fractions of *S. suis* 89/1591 (wild-type strain) from cultures grown at four different temperatures (37°C, 33°C, 28°C, and 23°C) and analyzed them by immunoblotting with anti-Sgp1 and anti-Sgp2 rabbit pAbs. Under all growth conditions, besides the signals corresponding to the predicted monomeric forms of Sgp1 and Sgp2 ($Sgp1_m$ and $Sgp2_m$, respectively), HMW ladders were detected by both anti-Sgp1 and anti-Sgp2 pAbs (Fig. 1B and Fig. 2A and B, lanes 1). The immunoreac-

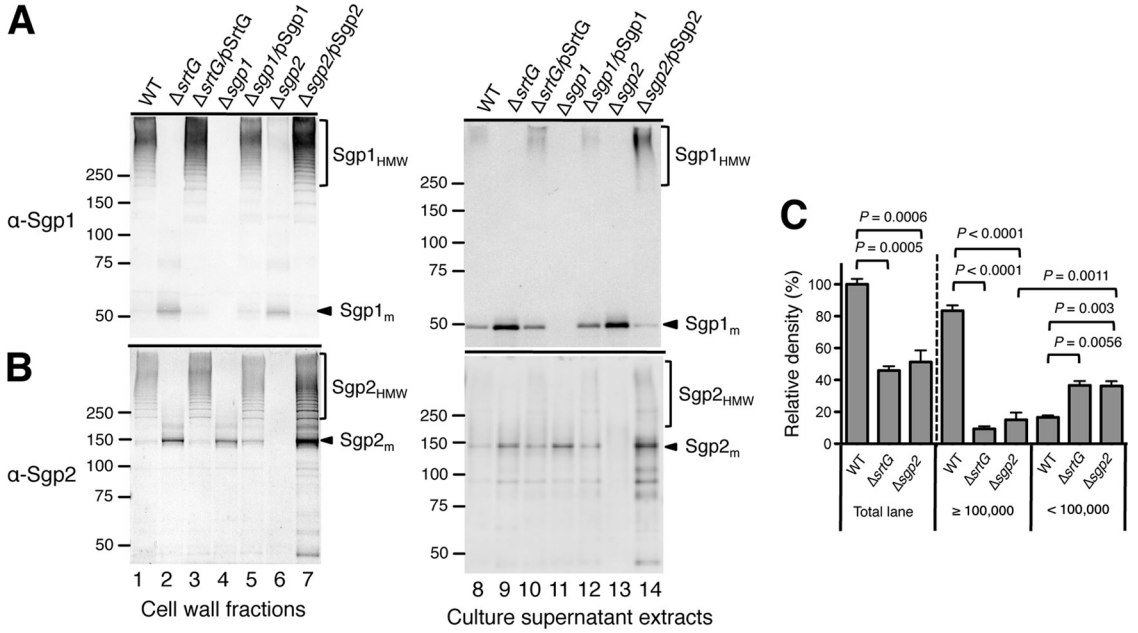

FIG. 2. Detection of polymerized subunits in cell wall fractions and culture supernatants by immunoblotting with anti-Sgp1 (α-Sgp1; A) or anti-Sgp2 (α-Sgp2; B) rabbit pAb. Immunoblotting was performed using the wild-type (WT) strain and isogenic derivatives (Δ*srtG*, Δ*sgp1*, and Δ*sgp2*). The strains were grown in THB at 28°C for 18 h. Each lane contains cell wall fractions extracted from cells in 500-μl cultures adjusted to an optical density at 600 nm of 0.6 or culture supernatants extracted from 500 μl of the adjusted cultures. In panels A and B, numbers to the left of the gels are molecular weights (in thousands). (C) Comparison of the relative density of reactive bands in the mixture of cell wall and culture supernatant fractions on immunoblots with anti-Sgp1 rabbit pAb. Cell wall and culture supernatant fractions of each strain were extracted from an equal volume of the culture adjusted to an optical density at 600 nm of 0.6 and mixed. The mixture corresponding to 500 μl of the adjusted culture was loaded in each lane and analyzed by immunoblotting. The sum of the density values of the bands within the total lane and lanes with molecular weights of ≥100,000 or <100,000 was calculated. Data were collected from five independent experiments, corrected by subtracting the density value of Δ*sgp1*, and are expressed as a percentage of the density values obtained in the total lane of the wild-type strain (mean ± standard deviation). Significant differences between two data are indicated by *P* values on the basis of Student's *t* test.

tive signals in bacterial samples grown at temperatures above 30°C were noticeably weaker than in those grown at temperatures below 30°C (Fig. 1B). Therefore, in subsequent experiments bacteria were grown at 28°C for pilus detection.

No immunoreactive signals of Sgp1 and Sgp2 were detected in the cell wall fractions extracted from Δ*sgp1* and Δ*sgp2* mutants, respectively (Fig. 2A, lane 4, and B, lane 6). The signals were restored by introduction of the intact genes into the respective mutants (Fig. 2A, lane 5, and B, lane 7). These results suggest that both putative subunits Sgp1 and Sgp2 are expressed and polymerized on the cell surface of strain 89/1591. Of note, disruption of one pilin subunit gene affected the polymerization of the other subunit (Fig. 2A, lane 6, and B, lane 4). These mutations were complemented by reintroduction of the respective genes (Fig. 2A, lane 7, and B, lane 5).

We also generated a Δ*srtG* mutant and investigated whether SrtG, like cognate sortases in the pilus clusters of other Gram-positive bacteria, is necessary for the polymerization of Sgp1 and Sgp2. In the cell wall fraction of the mutant, both $Sgp1_{HMW}$ and $Sgp2_{HMW}$ completely disappeared. Instead, $Sgp1_m$ and $Sgp2_m$ accumulated (Fig. 2A and B, lanes 2). Polymerization of the subunits was fully restored, and the signal intensities of $Sgp1_m$ and $Sgp2_m$ were reduced by introducing intact *srtG* into the mutant (Fig. 2A and B, lanes 3). These results suggest that SrtG is the specific pilin polymerase required for the polymerization of both Sgp1 and Sgp2.

Sgp1 and Sgp2 are components of the same pilus structure. To investigate whether the HMW polymers on the cell surface of the wild-type strain are composed of both Sgp1 and Sgp2, we performed immunoadsorption on the cell wall fractions using anti-Sgp1 or anti-Sgp2 rabbit pAb. Immunoprecipitated complexes were captured using protein G-coupled magnetic beads and were analyzed by immunoblotting using anti-Sgp1 mouse and anti-Sgp2 rabbit pAbs. Neither Sgp1 nor Sgp2 was detected in the cell wall fraction of the wild-type strain precipitated with preimmune rabbit serum of anti-Sgp1 or anti-Sgp2 pAb (data not shown). As expected, anti-Sgp1 pAb recovered both $Sgp1_{HMW}$ and $Sgp1_m$ from the wild-type strain (Fig. 3A, lane 1), but neither form was precipitated from the Δ*sgp1* mutant (Fig. 3A, lane 5). In addition, the anti-Sgp1 pAb coimmunoprecipitated $Sgp2_{HMW}$ from the wild-type strain (Fig. 3B, lane 1). Similarly, anti-Sgp2 pAb coimmunoprecipitated $Sgp1_{HMW}$ along with $Sgp2_{HMW}$ and $Sgp2_m$ from the wild-type strain (Fig. 3, lanes 2) but not from the Δ*sgp2* mutant (Fig. 3, lanes 6). These results indicate that Sgp1 and Sgp2 are components of the same pilus structure.

Electron microscopic evidence of pilus-like structures and localization of Sgp1 and Sgp2. To confirm the formation of fiber structures composed of Sgp1 and Sgp2, we carried out IEM with anti-Sgp1 mouse and anti-Sgp2 rabbit pAbs. IEM using anti-Sgp1 pAb showed the presence of appendages labeled by immunogold particles on the cell surface of the wild-

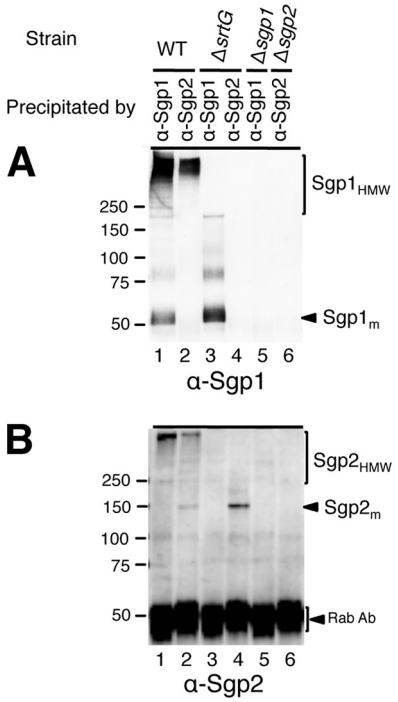

FIG. 3. Association of Sgp2 with Sgp1 in cell wall fractions. Cell wall fractions were extracted from cultures grown in THB at 28°C overnight. Each lane contains coimmunoprecipitated complexes of 0.1 μg anti-Sgp1 (α-Sgp1) or anti-Sgp2 (α-Sgp2) rabbit pAb and cell wall fractions extracted from cells in 1.5-ml cultures. The blot was probed with anti-Sgp1 mouse or anti-Sgp2 rabbit pAb. Rab Ab, reactive signals of α-Sgp1 or α-Sgp2 rabbit pAb used for coimmunoprecipitation. Numbers to the left of the gels are molecular weights (in thousands).

type strain. However, most of the surface appendages did not show the typical extended structure observed in pili of other Gram-positive bacteria (data not shown). We suspected that the thick polysaccharide capsule produced by strain 89/1591 may be cloaking pili, affecting their surface display and/or interfering with antibody binding. We thus constructed and examined the mutant carrying a disruption of the *cps2B* gene (mutant CPS2B; *cps2B*::pSET4s), which is necessary for capsule biosynthesis (34). Immunoblot analysis showed that this mutant expressed Sgp1 and Sgp2 (data not shown). On the cell surface of the mutant, typical pilus-like structures were clearly observed and the immunogold-labeled Sgp1 uniformly distributed along the structures (Fig. 4A). We then performed a double-labeling IEM experiment to show the localization of Sgp2 on the pilus structure. In the purified cell wall fraction, Sgp2 appeared to be located at one end of the structure (Fig. 4B). In addition, labeled Sgp2 was positioned both at the tip of Sgp1 pilus structures and on the cell surface of strain CPS2B (Fig. 4C). These results supported the localization of Sgp2 at the end of the pilus structure. In all experiments, preimmune sera of anti-Sgp1 and anti-Sgp2 pAbs did not label pilus fibers. Taken together, IEM and immunoblot analysis results demonstrate that Sgp1 is a major pilin subunit forming the pilus backbone, while Sgp2 is an ancillary subunit which appears to localize at one end of the structure.

Minor subunit Sgp2 is required for polymerization of major subunit Sgp1. Unlike the minor subunits of most well-characterized pili of Gram-positive bacteria, which were considered to be dispensable for polymerization of the major subunit (4, 10, 15, 29, 39), deletion of the gene encoding the minor subunit Sgp2 affected the polymerization of Sgp1 on the cell surface (Fig. 2A, lane 6). One possible explanation for these results might be that increased release of $Sgp1_{HMW}$ from the cell

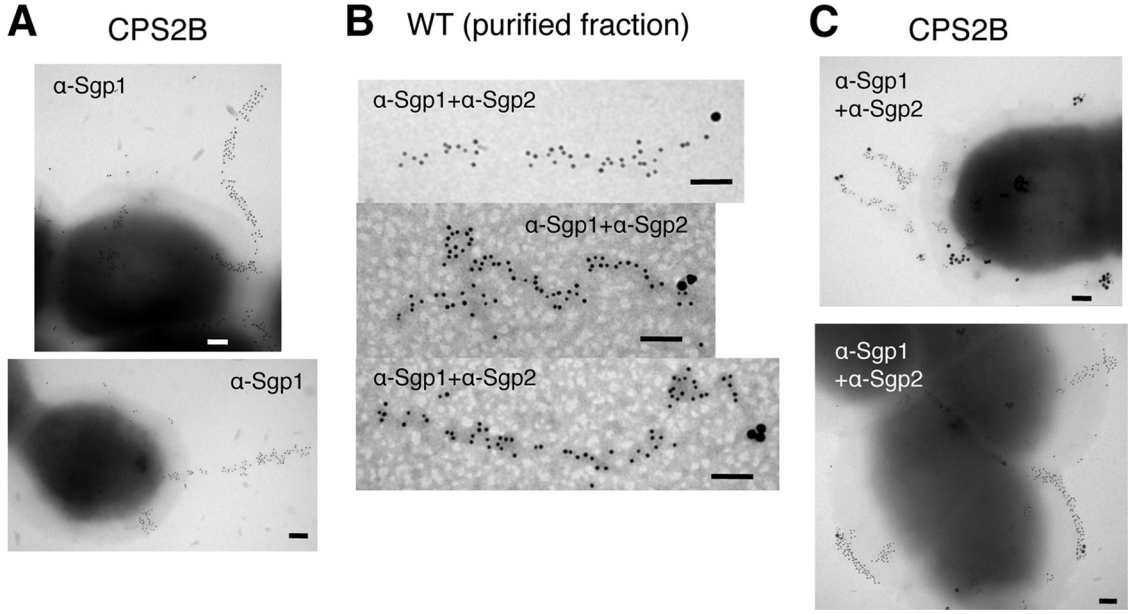

FIG. 4. Immunogold staining of pilus structures of *S. suis* strains. (A) Immunogold-labeled Sgp1 (gold particle size, 10 nm) on the cell surfaces of strain CPS2B. (B and C) Double immunogold labeling with mouse anti-Sgp1 pAbs (α-Sgp1; gold particle size, 10 nm) and rabbit anti-Sgp2 pAbs (α-Sgp2; gold particle size, 20 nm) in the partially purified cell wall fraction of the wild-type (WT) strain (B) and on the cell surface of strain CPS2B (C). Scale bars = 0.1 μm.

surface into the culture medium causes the reduction of cell surface $Sgp1_{HMW}$ in the Δ*sgp2* mutant. To test this hypothesis, we investigated culture supernatant fractions by immunoblotting. In the wild-type strain, both $Sgp1_{HMW}$ and $Sgp1_m$ were detected (Fig. 2A, lane 8). In the Δ*sgp2* mutant, the amount of $Sgp1_{HMW}$ released into the supernatant was noticeably lower than that observed in the wild-type strain (Fig. 2A, lane 13), but the amount of $Sgp1_m$ released was as high as that observed in the Δ*srtG* mutant (Fig. 2A, lanes 9 and 13). The amount of released $Sgp1_m$ diminished when intact *sgp2* was reintroduced into the mutant. In addition, in the complemented strain, the amount of released $Sgp1_{HMW}$ increased (Fig. 2A, lane 14). We also compared the density of anti-Sgp1-reactive bands in the mixtures of cell wall and culture supernatant fractions of the Δ*sgp2* mutant with the densities of the wild-type strain and the Δ*srtG* mutant. As shown in Fig. 2C, in the Δ*sgp2* mutant, the density of bands migrating at molecular weights of $<$100,000 was significantly higher than that of bands migrating at molecular weights of $\geq$100,000, accounting for more than 70% of the total density of all reactive bands. Similar results were obtained in the Δ*srtG* mutant. In contrast, in the wild-type strain, approximately 83% of the total density of all reactive bands was derived from the bands migrating at molecular weights of $\geq$100,000. In addition, the density of bands within molecular weights of $<$100,000 was 2.2-fold higher in the Δ*sgp2* mutant than in the wild-type strain and was the same as that in the Δ*srtG* mutant. Taken together, these results indicate that the weak signal of $Sgp1_{HMW}$ in the cell wall fraction of the Δ*sgp2* mutant (Fig. 2A, lane 6) was not caused by an increased release of Sgp1 polymers into the culture medium but reflected the abrogation of polymerization of Sgp1 in the absence of Sgp2. It is noteworthy that the total density of all reactive bands observed in the Δ*sgp2* and Δ*srtG* mutants was approximately only 50% of that observed in the wild-type strain. Because no signal was detected in the protoplast fractions of all the strains by immunoblotting (data not shown), it cannot be assumed that Sgp1 is retained in the protoplast of the mutants.

Sgp2 does not associate with Sgp1 in the absence of SrtG. We investigated whether Sgp2 associates with Sgp1 in the absence of cognate sortase SrtG by coimmunoprecipitation. From the cell wall fraction of the Δ*srtG* mutant, only $Sgp1_m$ and not $Sgp2_m$ was recovered by anti-Sgp1 pAb (Fig. 3, lanes 3). Similarly, $Sgp2_m$ but not $Sgp1_m$ was precipitated by anti-Sgp2 pAb (Fig. 3, lanes 4). Similar results were observed when the protoplast fraction was used (data not shown), suggesting that Sgp2 does not associate with Sgp1 in the absence of SrtG.

Sgp2 homologues encoded in other Gram-positive bacterial pilus gene clusters. BLAST analysis of Sgp2 showed that it had 32% identity with the fimbrial structural subunit protein FszD of *Streptococcus equi* subsp. *zooepidemicus* MGCS10565 and with four minor pilins of FCT-1 regions of *S. pyogenes* serotype M6 strains (MGAS10394, 2724, and 3650) and a serotype M23 strain (DSM 2071). Several regions of Sgp2 are highly conserved among these proteins (data not shown), and some of the regions also show significant similarities with hypothetical proteins of *Clostridium scindens*, *Peptoniphilus lacrimalis*, and four *Bifidobacterium* species (Fig. 5A). These hypothetical proteins contain CWSSs and are encoded in the close vicinity of putative class C sortase genes (Fig. 5B), suggesting that they are also pilus-associated proteins. Interestingly, these pilus and putative pilus gene clusters share a relatively simple genetic organization with the *srtG* cluster of *S. suis* (Fig. 5B). With the exception of the putative pilus gene clusters of *C. scindens* ATCC 35704 and *P. lacrimalis* 315-B, all of these clusters consist of a single sortase gene and two putative pilin subunit genes. Although one more CWSS-containing protein, PrtF1, is encoded in the FCT-1 region of *S. pyogenes* MGAS10394, it has been shown that PrtF1 is not incorporated into the pilus structure (21). In addition, these clusters do not encode signal peptidase homologues, which are considered to be important for pilus synthesis in *S. pyogenes* and *Streptococcus pneumoniae* (2, 23, 52).

DISCUSSION

The present study demonstrates that the *srtG* cluster of *S. suis* strain 89/1591 mediates the expression of pilus structures on the cell surface of this bacterium. Interestingly, our data show that in addition to the cognate sortase SrtG, the minor pilin subunit Sgp2 is required for the formation of the pilus backbone composed of major subunit Sgp1. To our knowledge, this is the first example of a minor pilin-mediated pilus polymerization system studied in detail.

In Gram-positive bacterial pili, minor pilin subunits are incorporated into the pilus, but their role in pilus assembly is poorly understood. In fact, several minor pilin subunits characterized in previous studies, such as SpaF and SpaG of *C. diphtheriae*, GBS52 and GBS104 of *Streptococcus agalactiae*, RrgA and RrgC of *S. pneumoniae*, and BcpB of *Bacillus cereus*, have been shown to be dispensable for the integrity or synthesis of the pilus backbone (4, 10, 15, 29, 39).

Recently, however, some minor pilin subunits were reported to contribute to pilus assembly. In *C. diphtheriae*, disruption of *spaB*, which encodes a minor pilin subunit of SpaA-type pili, resulted in a marked reduction of the amount of surface-associated major subunit polymers, and most of the synthesized polymers were not retained in the cell wall but were secreted into the culture medium (17). Similar results were observed when SpaI, a minor subunit of the SpaH-type pili of *C. diphtheriae*, and Spy0130, a minor subunit of the FCT-2 type pili of *S. pyogenes*, were absent. It was thus proposed that incorporation of these minor subunits into the pilus backbone serves as the terminal step in pilus polymerization and triggers concomitant cell wall linkage by the housekeeping sortase (17). In the *S. suis* Δ*sgp2* mutant, the amount of surface-associated major subunit polymers ($Sgp1_{HMW}$) was much lower than that in the wild-type strain. However, in contrast to the *spaB* mutant of *C. diphtheriae*, increased secretion of $Sgp1_{HMW}$ into the culture medium did not occur in the Δ*sgp2* mutant (Fig. 2A), indicating that Sgp2 is not involved in the termination of pilus polymerization. Instead, the Δ*sgp2* mutant showed increased amounts of $Sgp1_m$ both on the cell surface and in the culture medium, in a phenotype similar to that of the Δ*srtG* mutant (Fig. 2A and C).

Because reduction of the total amount of the major subunit protein Sgp1 was observed in the Δ*sgp2* mutant (Fig. 2C), it is conceivable that Sgp2 is involved in the stability of Sgp1 as a chaperone-like protein. In some Gram-negative bacterial pili, such as the type I and *pap* pili of *E. coli* (3, 36), the role of chaperone proteins in the stability of pilin proteins has been

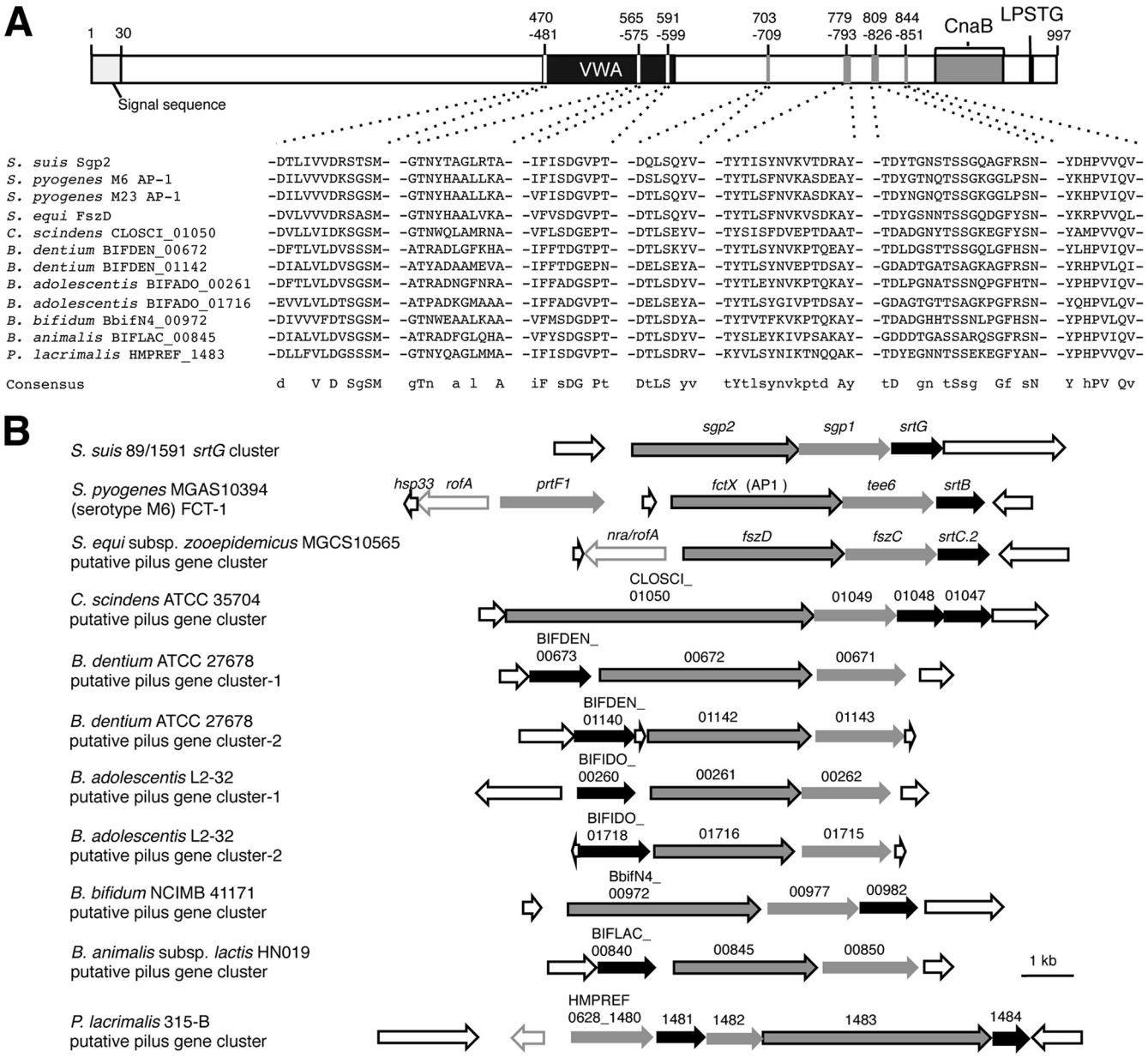

FIG. 5. (A) Schematic maps of Sgp2 and amino acid sequences conserved in Sgp2 and pilin and putative pilin subunits of several other Gram-positive bacteria. Sgp2 harbors an N-terminal signal sequence (amino acid positions 1 to 30), a von Willebrand factor type A (vWFA) domain (amino acid positions 468 to 601), a Cna protein B-type (CnaB) domain (amino acid positions 870 to 938), and a CWSS (LPSTG; amino acid positions 963 to 967). Alignment was carried out using the ClustalW program (45). Amino acid sequences conserved in all of the aligned sequences (in uppercase letters) or in more than seven sequences (in lowercase letters) were designated consensus sequences. The GenBank accession numbers of the listed proteins are as follows: ancillary protein 1 of *S. pyogenes* serotype M6 strains, AAL11466 (MGAS10394), ACH87890 (2724), and ACH87891 (3650); ancillary protein 1 of *S. pyogenes* M23 strain (DSM 2071), ACH87900; FszD of *S. equi* subsp. *zooepidemicus* MGCS10565, ACG63149; CLOSCI_01050 of *C. scindens* ATCC 35704, EDS08034; BIFDEN_00672 and BIFDEN_01142 of *B. dentium* ATCC 27678, EDT44861 and EDT45313, respectively; BIFADO_00261 and BIFADO_01716 of *B. adolescentis* L2-32, EDN83355 and EDN82663, respectively; BbifN4_00972 of *B. bifidum* NCIMB 41171, ZP_03645701; BIFLAC_00845 of *B. animalis* subsp. *lactis* HN019, EDT88620; and HMPREF0628_1483 of *P. lacrimalis* 315-B, EFA89995. (B) Genetic organizations of the *srtG* cluster, FCT-1 region, and putative pilus gene clusters containing *sgp2* homologues or genes partially homologous to *sgp2*. Black arrows, genes and putative genes encoding class C sortases; gray arrows, genes and putative genes encoding CWSS-containing proteins (*sgp2* and genes encoding proteins partially homologous to Sgp2 are outlined in black); open arrows outlined in gray, putative transcriptional regulator genes; open arrows outlined in black, other open reading frames. These genetic maps are constructed on the basis of the whole or draft genome sequences of the respective strains, and the GenBank accession numbers are as follows: *S. pyogenes* MGAS10394, CP000003; *S. equi* subsp. *zooepidemicus* MGCS10565, CP001129; *C. scindens* ATCC 35704, ABFY02000010; *B. dentium* ATCC 27678, ABIX02000002; *B. adolescentis* L2-32, AAXD02000018 and AAXD02000052; *B. bifidum* NCIMB 41171, ABQP01000004; *B. animalis* subsp. *lactis* HN019, ABOT01000008; and *P. lacrimalis* 315-B, ADDO01000052.

well studied. In these assembly pathways, named "chaperone-usher" pathways, the chaperone protein accelerates the folding of the pilin subunits and stabilizes them, and thus, the pilin proteins are degraded in the absence of the chaperone protein. In contrast, no chaperone protein had been implicated in the biogenesis of pili in Gram-positive bacteria. Recently, however, Zähner and Scott suggested a possible chaperone-like function of the type I signal peptidase homologue SipA2 in the T3 pilus formation of a *S. pyogenes* serotype M3 strain. In this strain, when *sipA2* was disrupted, the major subunit, T3, was not assembled as the subunits observed in the Δ*sgp2* mutant were (52). Although SipA2 did not act as a signal peptidase in the polymerization of T3, this protein seemed to interact with T3 independently of the presence of the pilin polymerase SrtC2, and thus, SipA2 was suggested to have a chaperone-like function in pilus formation (52). In our study, coimmunoprecipitation data indicated that Sgp2 does not associate with Sgp1 directly in the absence of SrtG. In addition, Sgp2 did not show any significant amino acid sequence similarities with SipA2 or the chaperone proteins of other species (data not shown). Therefore, it may be difficult to maintain that Sgp2 acts as a chaperone protein for Sgp1 by itself. We think that the reduction of Sgp1 may be due to the degradation of an excessive amount of unassembled monomers by proteinases. Alternatively, excessive amounts of $Sgp1_m$ may downregulate *sgp1* gene expression. In fact, the reduction of the major subunit protein was also observed in the Δ*srtG* mutant that expresses the Sgp2 protein (Fig. 2C), as well as in the cognate sortase gene mutants of other Gram-positive bacterial pili (13, 27). Taken together, our results suggest that Sgp2 participates in the polymerization of the major subunit itself.

The mechanism(s) by which Sgp2 contributes to the polymerization of the major subunit remains unclear. In the current models of pilus biogenesis, it is envisaged that pilin subunits are added to the base of the growing pilus by repeated transpeptidation reactions, until the terminal subunit is eventually linked covalently to the cell wall peptidoglycan. Pilin polymerization is driven by a pilus-specific class C sortase(s) encoded by genes clustered together with the genes encoding the pilin subunits, while attachment to the cell wall peptidoglycan is mediated by the housekeeping sortase (24, 38). Our IEM analysis showed that Sgp2 was localized at one end of the pilus fibers (Fig. 4B). In addition, labeled Sgp2 was observed both at the tip of the fibers and on the cell surface of the tested strain (Fig. 4C). Although the appearance of Sgp2 on the cell surface might be due to bent pili or pili lying across the bacterial surface, we hypothesize that Sgp2 located at the tip of the pilus represents an assembled minor subunit, while cell surface Sgp2 represents unassembled subunits that have been anchored to the cell wall as monomers by the housekeeping sortase SrtA. If this hypothesis is true, Sgp2 may act as a triggering signal for the cell to initiate the polymerization of Sgp1. Interestingly, as shown in Fig. 2, overexpression of Sgp2 but not that of major subunit Sgp1 resulted in an increase in the signal intensities of both $Sgp1_{HMW}$ and $Sgp2_{HMW}$. This result may support the possibility that Sgp2 is a trigger molecule to start pilus assembly.

To confirm the tip location of Sgp2 and to test the above possibility, elucidation of the Sgp1-Sgp2 linkage is necessary. The pilin polymerase catalyzes the formation of a peptide bond between the threonine (T) in the CWSS motif of one subunit and an ε-amino group of a lysine (K) in the next subunit (5, 12). Therefore, if Sgp2 is the tip pilin, the T in the CWSS motif of Sgp2 should bind to a K in Sgp1, as demonstrated in the linkage between the tip pilin Cpa and the backbone protein T3 of the *S. pyogenes* serotype M3 strain (27). In *C. diphtheriae* SpaA, SpaD, and SpaH pilins, the K residue in a conserved pilin motif (WXXXVXVYPK) participates in subunit linkage (10, 39, 47). Although most of the pilin proteins do not have a recognizable pilin motif in *S. pyogenes*, K161 in the backbone pilin Spy0128 of the serotype M1 strain has been identified as the residue that is linked to T of the CWSS motif of the next subunit (11). Recently, K173 in the T3 pilin of the serotype M3 strain, which corresponds to K161 of Spy0128, was also shown to be required both for T3 polymerization and for the attachment of Cpa to T3 (27). In *srtG* pili, however, no pilin motif is recognizable in either Sgp1 or Sgp2 (40). In addition, the K residue corresponding to the K residues identified in the *S. pyogenes* strains was not presented in Sgp1. Furthermore, conserved K residues were not found among Sgp1 and major and putative major subunits encoded in the pilus gene clusters listed in Fig. 5B (data not shown). Thus, we are currently pursuing experiments to identify the K residue required for Sgp1-Sgp2 linkage.

Unlike other well-characterized minor pilin subunits of Gram-positive bacterial pili, our data indicated the essential role of Sgp2 in pilus assembly. These differences may come from differences of the cognate sortases involved in pilus formation. However, SrtG does not contain any conserved domains other than the class C sortase domain and the catalytic active site (histidine, cysteine, and arginine residues) in the domain, which are conserved among pilus-forming sortases of Gram-positive bacteria. In addition, no conserved motif or domain specific to SrtG and cognate sortases in the pilus gene clusters encoding Sgp2 homologues was found by sequence alignment with other well-characterized class C sortases (data not shown). Recently, Manzano et al. analyzed the crystal structures of the pilus-forming sortases SrtC-1 and SrtC-3 of the pneumococcal *rlrA* islet and revealed the presence of a flexible lid that is absent from sortases not involved in pilus polymerization. Because the lid covered the substrate-recognition cleft in the pilus-forming sortases, it was proposed that this lid sterically blocks the active site and has a substrate-recognition gating function (19). As shown in Fig. S1 in the supplemental material, the sequences corresponding to the lid regions were also present in SrtG and the lid anchor residues (Asp-Pro/Ala-Hyd) were conserved, suggesting that SrtG also has a substrate-recognition gating function. Therefore, although we still do not have any evidence, it is tempting to speculate that only the monomeric form of Sgp2 and not that of Sgp1 can associate with SrtG, open the lid, and initiate pilus assembly. On the other hand, Sgp1 might need some conformational change to associate with SrtG, and the addition of Sgp2 or growing pilus to Sgp1 might drive the major pilin subunit recognizable by SrtG. Of note, Sgp1 and Sgp2 contain identical CWSSs (LPSTG), suggesting that the affinities of the substrate pentapeptides for the SrtG active site are comparable between Sgp1 and Sgp2.

As described above, we have identified the minor pilin subunit Sgp2, which is necessary for the assembly of *srtG* pili, and

discussed the possible role of Sgp2 as a triggering signal to initiate pilus assembly. Interestingly, phenotypes similar to those observed in the Δ*sgp2* mutant, i.e., reduction of major subunit polymers and increase of the monomeric form of the major subunit, were also observed when the minor pilin subunit-encoding gene in the FCT region of the *S. pyogenes* serotype M53 strain was disrupted (16). In addition, Sgp2 homologues are encoded in other Gram-positive bacterial pilus clusters, and the clusters share a relatively simple genetic organization with the *srtG* cluster of *S. suis* (Fig. 5). Therefore, the participation of the minor subunits in polymerization of the pilus backbone may not be unique to *srtG* pili but may be common to some types of pili.

In many Gram-positive bacterial pathogens, pili have been reported to contribute to the adherence to and invasion of host cells and to biofilm formation (18, 26, 33, 44). The biological significance of *srtG* cluster-mediated pili is unknown. However, several *S. suis* isolates from human patients and diseased pigs possessed all genes in the *srtG* cluster (40). In addition, most of the isolates expressed at least $Sgp1_{HMW}$ on the cell surface (unpublished results). Our data show that *srtG* pili are expressed at higher levels when bacteria are grown at temperatures of less than 30°C. Interestingly, the surface temperatures of different external body parts of pigs (snout, ears, vertex, back, and flank) ranged from 20°C to 30°C when the environmental temperature was approximately 20°C (unpublished observations). These findings suggest that this pilus may be important for the interaction of *S. suis* with the surface structures of host animals. It is noteworthy that, similar to the *srtG* pilus, the expression of the major subunit FctA of the FCT-3 pili in *S. pyogenes* serotype M49 also increased with lower temperatures (23). It was suggested that the expression of FctA may be regulated by a bistability mode. In fact, at 37°C, only 20% of all cells express FctA, while at 30°C, approximately 50% of a given population does so (23). Currently, the regulatory mechanism of *srtG* pilus expression is not known. Further detailed investigations of the mode and molecular background of the temperature-dependent expression of the pili may provide clues to understand the biological advantage of *srtG* pilus expression by *S. suis*.

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REFERENCES

1. **Arends, J. P., and H. C. Zanen.** 1988. Meningitis caused by *Streptococcus suis* in humans. Rev. Infect. Dis. **10:**131–137.
2. **Bagnoli, F., et al.** 2008. A second pilus type in *Streptococcus pneumoniae* is prevalent in emerging serotypes and mediates adhesion to host cells. J. Bacteriol. **190:**5480–5492.
3. **Barnhart, M. M., et al.** 2000. PapD-like chaperones provide the missing information for folding of pilin proteins. Proc. Natl. Acad. Sci. U. S. A. **97:**7709–7714.
4. **Budzik, J. M., L. A. Marraffini, and O. Schneewind.** 2007. Assembly of pili on the surface of *Bacillus cereus* vegetative cells. Mol. Microbiol. **66:**495–510.
5. **Budzik, J. M., et al.** 2008. Amide bonds assemble pili on the surface of bacilli. Proc. Natl. Acad. Sci. U. S. A. **105:**10215–10220.
6. **Casadaban, M. J., and S. N. Cohen.** 1980. Analysis of gene control signals by DNA fusion and cloning in *Escherichia coli*. J. Mol. Biol. **138:**179–207.
7. **Chang, B., et al.** 2006. Characteristics of *Streptococcus suis* isolated from patients in Japan. Jpn. J. Infect. Dis. **59:**397–399.
8. **Dramsi, S., P. Trieu-Cuot, and H. Bierne.** 2005. Sorting sortases: a nomenclature proposal for the various sortases of Gram-positive bacteria. Res. Microbiol. **156:**289–297.
9. **Fittipaldi, N., M. Gottschalk, G. Vanier, F. Daigle, and J. Harel.** 2007. Use of selective capture of transcribed sequences to identify genes preferentially expressed by *Streptococcus suis* upon interaction with porcine brain microvascular endothelial cells. Appl. Environ. Microbiol. **73:**4359–4364.
10. **Gaspar, A. H., and H. Ton-That.** 2006. Assembly of distinct pilus structures on the surface of *Corynebacterium diphtheriae*. J. Bacteriol. **188:**1526–1533.
11. **Kang, H. J., F. Coulibaly, F. Clow, T. Proft, and E. N. Baker.** 2007. Stabilizing isopeptide bonds revealed in gram-positive bacterial pilus structure. Science **318:**1625–1628.
12. **Kang, H. J., N. G. Paterson, A. H. Gaspar, H. Ton-That, and E. N. Baker.** 2009. The *Corynebacterium diphtheriae* shaft pilin SpaA is built of tandem Ig-like modules with stabilizing isopeptide and disulfide bonds. Proc. Natl. Acad. Sci. U. S. A. **106:**16967–16971.
13. **Konto-Ghiorghi, Y., et al.** 2009. Dual role for pilus in adherence to epithelial cells and biofilm formation in *Streptococcus agalactiae*. PLoS Pathog. **5:**e1000422.
14. **Lee, G. T., et al.** 2008. *Streptococcus suis* meningitis, United States. Emerg. Infect. Dis. **14:**183–185.
15. **LeMieux, J., S. Woody, and A. Camilli.** 2008. Roles of the sortases of *Streptococcus pneumoniae* in assembly of the RlrA pilus. J. Bacteriol. **190:**6002–6013.
16. **Lizano, S., F. Luo, and D. E. Bessen.** 2007. Role of streptococcal T antigens in superficial skin infection. J. Bacteriol. **189:**1426–1434.
17. **Mandlik, A., A. Das, and H. Ton-That.** 2008. The molecular switch that activates the cell wall anchoring step of pilus assembly in gram-positive bacteria. Proc. Natl. Acad. Sci. U. S. A. **105:**14147–14152.
18. **Mandlik, A., A. Swierczynski, A. Das, and H. Ton-That.** 2008. Pili in Gram-positive bacteria: assembly, involvement in colonization and biofilm development. Trends Microbiol. **16:**33–40.
19. **Manzano, C., et al.** 2008. Sortase-mediated pilus fiber biogenesis in *Streptococcus pneumoniae*. Structure **16:**1838–1848.
20. **McNab, R., and H. F. Jenkinson.** 1998. Lipoproteins and other cell-surface associated proteins in streptococci. Methods Cell Sci. **20:**209–216.
21. **Mora, M., et al.** 2005. Group A *Streptococcus* produce pilus-like structures containing protective antigens and Lancefield T antigens. Proc. Natl. Acad. Sci. U. S. A. **102:**15641–15646.
22. **Munier, H., et al.** 1991. Isolation and characterization of catalytic and calmodulin-binding domains of *Bordetella pertussis* adenylate cyclase. Eur. J. Biochem. **196:**469–474.
23. **Nakata, M., et al.** 2009. Mode of expression and functional characterization of FCT-3 pilus region-encoded proteins in *Streptococcus pyogenes* serotype M49. Infect. Immun. **77:**32–44.
24. **Nobbs, A. H., et al.** 2008. Sortase A utilizes an ancillary protein anchor for efficient cell wall anchoring of pili in *Streptococcus agalactiae*. Infect. Immun. **76:**3550–3560.
25. **Osaki, M., D. Takamatsu, N. Tsuji, and T. Sekizaki.** 2000. Cloning and characterization of the gene encoding O-acetylserine lyase from *Streptococcus suis*. Curr. Microbiol. **40:**67–71.
26. **Proft, T., and E. N. Baker.** 2009. Pili in Gram-negative and Gram-positive bacteria—structure, assembly and their role in disease. Cell. Mol. Life Sci. **66:**613–635.
27. **Quigley, B., D. Zähner, M. Hatkoff, D. Thanassi, and J. Scott.** 2009. Linkage of T3 and Cpa pilins in the *Streptococcus pyogenes* M3 pilus. Mol. Microbiol. **72:**1379–1394.
28. **Rogé, J., and J. M. Betton.** 2005. Use of pIVEX plasmids for protein overproduction in *Escherichia coli*. Microb. Cell Fact. **4:**18.
29. **Rosini, R., et al.** 2006. Identification of novel genomic islands coding for antigenic pilus-like structures in *Streptococcus agalactiae*. Mol. Microbiol. **61:**126–141.
30. **Salasia, S. I., C. Lämmler, and G. Herrmann.** 1995. Properties of a *Streptococcus suis* isolate of serotype 2 and two capsular mutants. Vet. Microbiol. **45:**151–156.
31. **Sambrook, J., and D. W. Russell.** 2001. Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
32. **Schneewind, O., P. Model, and V. A. Fischetti.** 1992. Sorting of protein A to the staphylococcal cell wall. Cell **70:**267–281.
33. **Scott, J. R., and D. Zähner.** 2006. Pili with strong attachments: Gram-positive bacteria do it differently. Mol. Microbiol. **62:**320–330.
34. **Smith, H., et al.** 1999. Identification and characterization of the cps locus of *Streptococcus suis* serotype 2: the capsule protects against phagocytosis and is an important virulence factor. Infect. Immun. **67:**1750–1756.
35. **Smith, W., et al.** 2010. Roles of minor pilin subunits Spy0125 and Spy0130 in the M1 *Streptococcus pyogenes* strain SF370. J. Bacteriol. **192:**4651–4659.
36. **Soto, G. E., and S. J. Hultgren.** 1999. Bacterial adhesins: common themes and variations in architecture and assembly. J. Bacteriol. **181:**1059–1071.

37. **Staats, J. J., I. Feder, O. Okwumabua, and M. M. Chengappa.** 1997. *Streptococcus suis*: past and present. Vet. Res. Commun. **21:**381–407.
38. **Swaminathan, A., et al.** 2007. Housekeeping sortase facilitates the cell wall anchoring of pilus polymers in *Corynebacterium diphtheriae*. Mol. Microbiol. **66:**961–974.
39. **Swierczynski, A., and H. Ton-That.** 2006. Type III pilus of corynebacteria: pilus length is determined by the level of its major pilin subunit. J. Bacteriol. **188:**6318–6325.
40. **Takamatsu, D., et al.** 2009. Genetic organization and preferential distribution of putative pilus gene clusters in *Streptococcus suis*. Vet. Microbiol. **138:**132–139.
41. **Takamatsu, D., M. Osaki, and T. Sekizaki.** 2001. Construction and characterization of *Streptococcus suis-Escherichia coli* shuttle cloning vectors. Plasmid **45:**101–113.
42. **Takamatsu, D., M. Osaki, and T. Sekizaki.** 2001. Thermosensitive suicide vectors for gene replacement in *Streptococcus suis*. Plasmid **46:**140–148.
43. **Takamatsu, D., et al.** 2008. *Streptococcus suis* in humans, Thailand. Emerg. Infect. Dis. **14:**181–183.
44. **Telford, J. L., M. A. Barocchi, I. Margarit, R. Rappuoli, and G. Grandi.** 2006. Pili in Gram-positive pathogens. Nat. Rev. Microbiol. **4:**509–519.
45. **Thompson, J. D., D. G. Higgins, and T. J. Gibson.** 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. **22:**4673–4680.
46. **Ton-That, H., and O. Schneewind.** 2004. Assembly of pili in Gram-positive bacteria. Trends Microbiol. **12:**228–234.
47. **Ton-That, H., and O. Schneewind.** 2003. Assembly of pili on the surface of *Corynebacterium diphtheriae*. Mol. Microbiol. **50:**1429–1438.
48. **Warrens, A. N., M. D. Jones, and R. I. Lechler.** 1997. Splicing by overlap extension by PCR using asymmetric amplification: an improved technique for the generation of hybrid proteins of immunological interest. Gene **186:** 29–35.
49. **Wertheim, H. F., et al.** 2009. *Streptococcus suis*, an important cause of adult bacterial meningitis in northern Vietnam. PLoS One **4:**e5973.
50. **Yanagawa, R., and K. Otsuki.** 1970. Some properties of the pili of *Corynebacterium renale*. J. Bacteriol. **101:**1063–1069.
51. **Ye, C., et al.** 2006. *Streptococcus suis* sequence type 7 outbreak, Sichuan, China. Emerg. Infect. Dis. **12:**1203–1208.
52. **Zähner, D., and J. R. Scott.** 2008. SipA is required for pilus formation in *Streptococcus pyogenes* serotype M3. J. Bacteriol. **190:**527–535.