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Evidence for Lateral Transfer of the Suilysin Gene Region of *Streptococcus suis*

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Suilysin is a cholesterol-binding cytolysin encoded by *sly* in *Streptococcus suis*. DNA sequence determination of the *sly* locus in a strain lacking *sly* revealed the presence of another gene, designated *orf102*, in the place of *sly*. No transposable element or long-repeat sequence was found in the close vicinity. Except for six strains whose corresponding loci have been rearranged, all of the remaining 62 strains examined had either *sly* or *orf102* at the same locus and their flanking regions were conserved. The genetic organizations having either *sly* or *orf102* were found in the strains whose 16S rRNA sequences were identical. These results suggest that *S. suis* acquired *sly* or *orf102* from a foreign source and that these genes subsequently spread among *S. suis* strains by homologous recombination.

Streptococcus suis is a gram-positive coccus that has been identified as a cause of meningitis, septicemia, arthritis, and sudden death in pigs (6). It can also cause human meningitis (2, 22). Thirty-five capsular serotypes have been described so far (11, 12, 17, 27), and some serotypes, especially serotype 2, are more frequently isolated from diseased pigs than others (6, 15, 16). However, not all strains of *S. suis* serotype 2 are virulent and there is variation in the degrees of virulence among the strains (40, 42). Comparisons between virulent and avirulent strains of *S. suis* have led to the proposal of several cellular and extracellular components as candidates for virulence markers (34, 41, 42). However, there are several variants of these markers and some *S. suis* isolates from diseased pigs do not possess one or more of them (1, 3, 4, 13, 19, 38, 41), indicating genetic heterogeneity with respect to these markers. Recently, it was shown that some *S. suis* strains possess a type II restriction-modification (R-M) system, designated *Ssu*DAT1I, which is an isoschizomer of *Moraxella bovis Mbo*I (10), whereas some other strains lack the system (32). Nucleotide sequence comparison between strains having the *Ssu*DAT1I system and those lacking the system revealed that the *Ssu*DAT1I system was originally inserted into the *S. suis* chromosome from a foreign source by illegitimate recombination and was subsequently transferred among *S. suis* strains by homologous recombination (31, 32). These findings raise the question of whether a series of genetic exchanges, exemplified by the *Ssu*DAT1I system, also occurred in other genes and is one of the typical processes involved in the evolution of this bacterium, which constitutes a population containing strains with various combinations of virulence markers.

Some strains of *S. suis* produce a hemolysin called suilysin, which is a member of the family of cholesterol-binding cytolysins (alternatively called thiol-activated cytolysins) (8, 14, 18). A gene encoding suilysin (*sly*) has been cloned and sequenced (30), and the absence of *sly* in some *S. suis* strains was demonstrated by PCR using different sets of primers and/or by Southern hybridization analysis using cloned or amplified *sly* as a probe (24, 30). In this study, using 40 field isolates and 28 serotype reference strains, we analyzed the *sly* region and the corresponding chromosomal region of the strains lacking *sly* in order to provide additional knowledge about the acquisition and intraspecies transfer of genes in this bacterium.

The *S. suis* strains used in this study are listed in Table 1. The *Escherichia coli* strains used were XL1 Blue MRF′ (Stratagene, La Jolla, Calif.), XLOLR (Stratagene), and DH5α (29). *S. suis* strains were grown in Todd-Hewitt broth or agar medium (Difco Laboratories, Detroit, Mich.) supplemented with 2% yeast extract at 37°C under 5% CO_2. *E. coli* strains were cultured in Luria-Bertani broth or agar medium (Difco Laboratories) supplemented, when necessary, with ampicillin (50 μg/ml) and kanamycin (25 μg/ml) at 37°C. On the basis of our previous data (37), the sequences of the 5,545-bp *sly* region of strain DAT2 and the corresponding 4,257-bp chromosomal region of strain DAT1, which lacks the *sly* gene, were determined. The sequences were searched against current DNA databases by using either the blastn, blastp, blastx, tblastn, or tblastx program network services available at the National Center for Biotechnology Information, Bethesda, Md. (http://www.ncbi.nlm.nih.gov/). Further DNA comparisons were made with the preliminary sequence data released by genome sequencing projects at various institutions (University of Oklahoma, Norman, Okla. [http://www.genome.ou.edu/smutans.html]; Université Catholique de Louvain, Louvain-la-Neuve, Belgium [http://www.biol.ucl.ac.be/gene/genome/blast.html]; The Sanger Centre, Cambridge, United Kingdom [http://www.sanger.ac.uk/Projects/S_equi/]; The Institute for Genomic Research, Rockville, Md. [http://www.tigr.org/tdb/s_gordonii.shtml]).

The *sly* region of strain DAT2 contained five putative open reading frames (ORFs) (Fig. 1). The five ORFs found in this region were carried on the same DNA strand. Two ORFs were located upstream of *sly*. The first ORF, designated ORF100, encoded a 148-amino-acid protein whose N-terminal end was truncated. The protein showed 65% identity with an ABC transporter homolog of *Bacillus subtilis* (accession no.

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TABLE 1. Strains of *S. suis* used and the genetic organizations of their *sly* loci

Type of genetic organization[a]	Strain	Serotype	Reference
DAT1	Field isolates		
	211, 212	1	32
	NIAH11318	1/2	31
	DAT1, 194, 195, 196, 197, 198, 199, 200, 202, 205, 220, 221, 222, 226, 227, 229, 230, 233, 234, 235, 236, 238, 239, 243, 244	2	32, 35
	Reference strains		
	4961	3	27
	2524	6	27
	8074	7	27
	22083	9	12
	4417	10	12
	12814	11	12
	8830	12	12
	2726	16	12
	89-3576-3	25	11
	89-5259	27	11
DAT2	Field isolates		
	203, 204	1	32
	DAT2, 193, 207, 209, 210, 213, 223, 228, 246, 247	2	32, 36
	Reference strains		
	NCTC10237	1	27
	NCTC10234	2	27
	6407	4	27
	11538	5	27
	14636	8	27
	13730	14	12
	NCTC10446	15	12
	93A	17	12
	NT77	18	12
	42A	19	12
	89-2479	23	11
	89-590	28	11
Atypical	Reference strains		
	10581	13	12
	86-5192	20	12
	14A	21	12
	88-1861	22	12
	88-5299A	24	11
	89-4109-1	26	11

[a] Classification of the strains into the DAT1, DAT2, and atypical types was based on the results of PCR amplification and Southern hybridization as described in the text.

H69828). The second ORF, designated ORF101, encoded a 236-amino-acid protein which showed 33% identity with a conserved hypothetical protein of *Streptococcus pyogenes* (accession no. AAK33575), but the gene was not preceded by a typical Shine-Dalgarno (SD) sequence. The ORF just downstream of *sly* encoded a 233-amino-acid protein which showed 73% identity with a putative *N*-acetylmannosamine-6-phosphate epimerase of *S. pyogenes* (accession no. AAK33327), and the gene was designated *nanE*. The remaining ORF encoded a 403-amino-acid protein whose C-terminal end was truncated. The protein showed 56% identity with phosphotransferase system II BC components of *S. pneumoniae* (accession no. AAK75763), and the gene was designated *ptsG*. The *sly*, *nanE*, and *ptsG* genes were not preceded by a typical SD sequence, although a conserved sequence, 5′-GAAAGGA-3′, was located 8 or 9 bp upstream of the putative start codons. The genes identified in this region were thus organized as shown in Fig. 1, and this genetic organization was designated the DAT2 type. The genetic organization of the DAT2 type was different from those of the pneumolysin gene (*ply*) region of *S. pneumoniae* strain TIGR4 (39) and the streptolysin O gene (*slo*) region of *S. pyogenes* strain SF370 (9). On the other hand, four genes, *orf100*, *orf101*, *nanE*, and *ptsG*, were also present in the corresponding chromosomal region of strain DAT1, although the *orf101* homolog of strain DAT1 was 15 bp shorter than *orf101* of strain DAT2. However, a putative ORF, designated ORF102, which was completely different from *sly*, was found in the place of *sly*, and thus *sly* was completely missing from strain DAT1. ORF102 encoded a 194-amino-acid protein which showed 70% identity with a conserved hypothetical protein of *S. pneumoniae* (accession no. AAK74572). The genes identified in this region were ordered as shown in Fig. 1, and the genetic organization was designated the DAT1 type.

Nucleotide sequence comparison between DAT2- and DAT1-type organizations revealed that left- and right-hand ends of the regions were highly conserved (more than 98% identity), whereas the central regions were diverse (Fig. 1). *sly* and *orf102* were bounded by regions which showed relatively low homologies (65.9 and 66.5% identities) and constituted mosaic structures with low- and high-homology segments (Fig. 1 and 2). The genetic regions with relatively low homologies overlapped the 3′ region of *orf101* and the 5′ end of *nanE* (Fig. 1 and 2). The average G+C contents of *sly* (39.2%) and *orf102* (43.6%), as well as those of other regions, were similar to that of the total genome of *S. suis* (39 to 41%) (20), whereas *sly* and *orf102* were encompassed by segments of remarkably low G+C contents, and one segment located downstream of the genes coincided with a relatively low-homology region (Fig. 1). The codon usage patterns for the *sly* and *orf102* genes were not anomalous compared to those previously reported for purine and cysteine biosynthetic genes (26, 32). No transposable element or long-repeat sequence was found in the 5,545 or 4,257-bp sequence. However, a 109-bp segment, which was similar to repeated DNA elements (BOX elements) found in *S. pneumoniae* (21, 23), was located 99 bp downstream of *orf102* (Fig. 2). It was recently shown that similar DNA elements were located in the vicinity of genes encoding sortase-like proteins in *S. suis* strain NCTC10234 (25). The 109-bp segment located downstream of *orf102* was one such homolog, suggesting that the BOX elements are scattered throughout the genome of *S. suis*, as was observed in *S. pneumoniae* (39). While one of the BOX elements was located downstream of *ply* in *S. pneumoniae* (23), no BOX element was found downstream of *sly*.

For the characterization of 68 *S. suis* strains with respect to the genetic organization of their *sly* loci, DNA fragments were amplified from the genomic DNAs of these strains by PCR with primers OS1 and OS2, which were complementary to highly conserved sequences in the *sly*-flanking regions (Fig. 1 and Table 2). The conditions of the PCR were essentially the same as described previously (36). The PCR products were analyzed by Southern hybridization with the *sly* and *orf102* probes by procedures described previously (32), except that hybridization was carried out at 68°C. Genomic Southern hy-

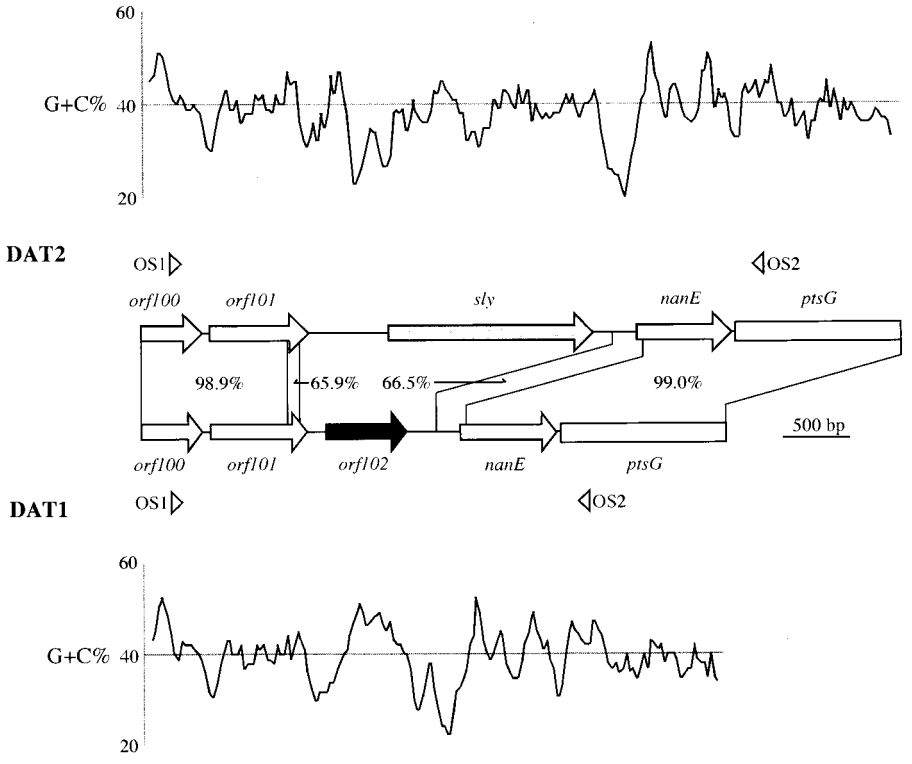

FIG. 1. Physical and genetic maps of the 5,519- and 4,232-bp chromosomal regions of *S. suis* DAT2 and DAT1, respectively, with putative genes indicated by arrows and boxes. Regions with high and relatively low identities are represented by lines drawn between the two physical maps, and the percentages of nucleotide identity are indicated in the spaces between the physical maps. In the line graphs, G+C contents scanned with a sliding window of 100 bp are shown in 25-bp increments; arrowheads between the line graphs and the physical maps depict the positions of primers used for PCR. The chromosomal regions shown correspond to nucleotides 1 to 5,519 and 26 to 4,257 of the sequences with accession no. AB055649 (DAT2) and AB071359 (DAT1), respectively.

bridization was also performed using *S. suis* DNAs that had been digested with *Pst*I, for which no cutting site is present in the *sly* or *orf102* regions. For the preparation of *sly* and *orf102* probes, the *sly* gene region was amplified from the genomic DNA of DAT2 with primers SL1 and SL4 (Table 2) and the *orf102* gene region was amplified from the genomic DNA of DAT1 with primers ORF102-1 and ORF102-2 (Table 2), both of which were followed by cloning into pCR2.1 (Invitrogen, Groningen, The Netherlands). A 3.0-kb fragment was amplified with primers OS1 and OS2 from the genomic DNAs of 28 field isolates, including strain DAT1, as well as 10 reference strains. The amplified fragments were hybridized with the *orf102* probe but not with the *sly* probe (data not shown). A DNA fragment that hybridized with the *orf102* probe was also seen in the digested DNAs of the 28 field isolates and 10 reference strains, but no hybridizing fragment was seen when the *sly* probe was used (data not shown), indicating that the DAT1-type genetic organization was conserved in these strains (Table 1). On the other hand, a 4.3-kb fragment was amplified from genomic DNAs of the remaining 12 field isolates, including strain DAT2, as well as 12 reference strains (data not shown). The amplified fragments and a DNA fragment of the digested DNAs from the 12 field isolates and 12 reference strains were hybridized with the *sly* probe but not with the *orf102* probe (data not shown), indicating that the DAT2-type genetic organization was conserved in these strains (Table 1). However, no DNA fragment was amplified with primers OS1 and OS2 from the genomic DNAs of the remaining six reference strains of serotypes 13, 20, 21, 22, 24, and 26. The genomic DNAs of these strains did not show a hybridizing fragment with the *sly* probe (data not shown). The strains of serotypes 13, 21, and 24 provided a DNA fragment that hybridized with the *orf102* probe, although the hybridization signal in the strain of serotype 24 was weak (Fig. 3). No fragment hybridizing with the *orf102* probe appeared in the digested DNAs of the remaining three reference strains of serotypes 20, 22, and 26 (Fig. 3). These results indicate that the six reference strains had different genetic organizations with respect to the *sly* locus, and the six strains were collectively grouped into the atypical type of genetic organization (Table 1). Genomic Southern hybridization and PCR with various combinations of probes and primers (Fig. 3) were performed to examine the genetic organizations of the six atypical strains. As summarized in Fig. 3, the results indicated that genetic rearrangements in the *sly* loci had occurred in these strains. Consequently, six atypical-type strains could be divided into four minor types (Fig. 3); their genetic organizations are represented in Fig. 4. The *sly* and *orf102* genes of several selected strains were amplified by PCR with primers SD1 and SD2 (Table 2) and directly sequenced. Comparison of the *sly* sequences among strains DAT2 and 203 and reference strains of serotypes 1, 4, 8, 19, 23, and 28 showed striking similarities (99.4 to 100% identity), and the deduced amino acid sequences were completely identical with the exception of one amino acid difference found in the serotype 1

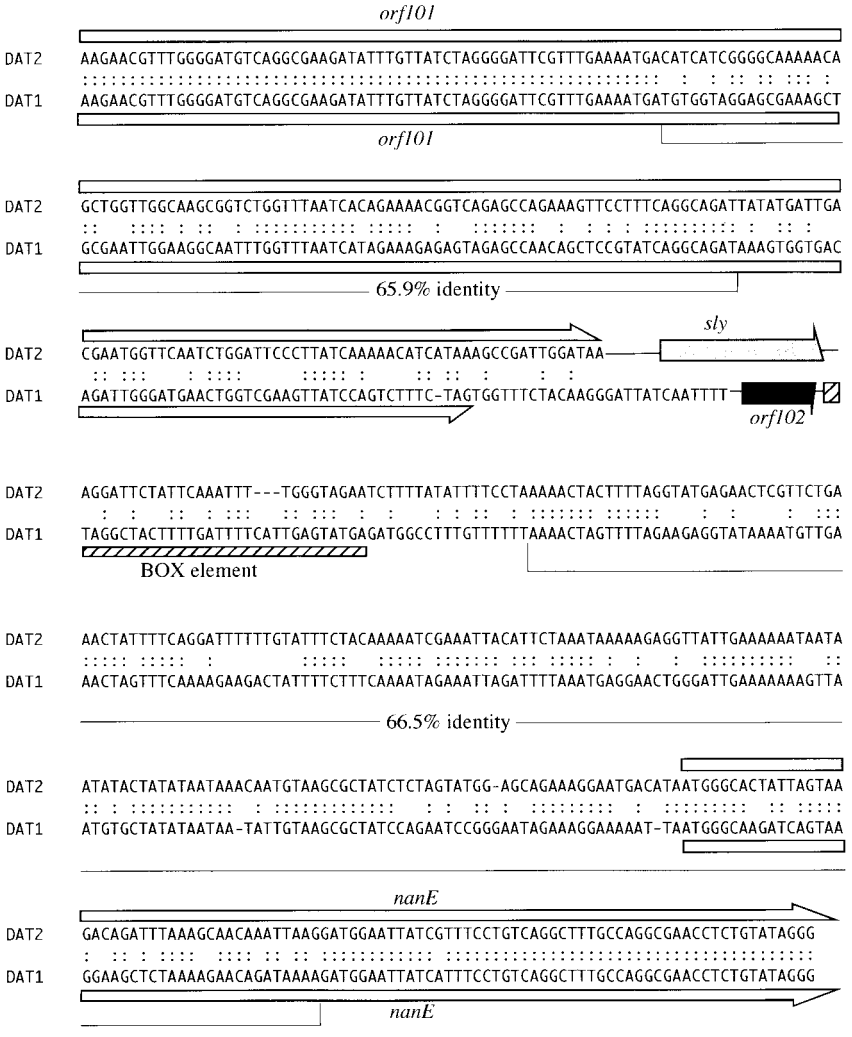

FIG. 2. Nucleotide sequence comparison between *sly*-flanking region of DAT2 and *orf102*-flanking region of DAT1. Relatively low-homology regions and the percentages of nucleotide identity are indicated below the sequences. Colons, identical nucleotides; open boxes and arrows, *orf101* and *nanE*; gray and black arrows, *sly* and *orf102*, respectively; hatched box, BOX element.

reference strain. On the other hand, the identities among the *orf102* sequences of strains DAT1 and 226 and reference strains of serotypes 6, 7, 9, and 12 ranged from 96.1 to 100% and several amino acid differences occurred among the ORF102 proteins of these strains.

A phylogenetic tree was constructed on the basis of the sequence discrepancies in the 16S rRNA genes of 15 field isolates and reference strains of serotypes 1 to 28. The 16S rRNA genes of the 15 field isolates of *S. suis* were amplified using the previously reported primers F1 and R13 (Table 2) (7) and sequenced. The 16S rRNA gene sequences retrieved from the GenBank database were for *S. pyogenes* NCDO2381 (accession no. X59029) and *S. suis* reference strains of serotypes 1 and 2 to 28 (accession no. AF009475 and AF009477 to AF009503, respectively). The tree was constructed by using CLUSTAL W (http://www.ddbj.nig.ac.jp/E-mail/clustalw-e.html) and the programs of the Phylogeny Inference Package (PHYLIP, version 3.573c, 1995) as described previously (5), except that sequence similarities for *S. suis* and *S. pyogenes* strains were determined only for a region corresponding to nucleotides 28 to 1,473 of the *E. coli* 16S rRNA sequence, and unambiguous parts of the aligned sequence were cut and concatenated to a single data matrix. The topology of the tree obtained generally resembles that of the previously reported tree (5). *S. suis* strains were divided into three clusters on the basis of their distances from the 16S rRNA sequence of the serotype 1 reference strain, as previously performed by Chatellier et al. (5). Strain 226 and the reference strains of serotypes 7 and 9 belonged to cluster II (distances between 0.0126 and 0.0190). Reference strains of serotypes 20, 22, and 26 belonged to cluster III (distances between 0.0219 and 0.0291). Other *S. suis* strains were grouped into cluster I (distances between 0 and 0.0105) (Fig. 4). From these results, there were four principal findings. First, there were both DAT1- and DAT2-type genetic organizations among the strains in which the 16S rRNA sequences were identical (e.g., reference strains of serotypes 12 and 14 or 6 and 18). Second, all the strains of DAT2-type genetic organization were classified into cluster I, whereas strains of DAT1-type genetic organization were widely dis-

TABLE 2. Oligonucleotide primers used

Primer	Sequence (5′-3′)	Location or description
OS1	AAGCAACTTCTCATATTGATACGGAGACGG	5′ region of truncated *orf100*
OS2	CCACGCTTGATCCAATACAGGAAATTGTGC	5′ region of truncated *ptsG*
SL1	TACATTGATAATCCGCCAGC	5′ region of *sly*
SL4	AAACTGTTCTCCACCATTCC	3′ region of *sly*
ORF102-1	ACGAGAAAACCTTGCGACTG	5′ region of *orf102*
ORF102-2	CTGGATTGATAGGAGTGTTG	3′ region of *orf102*
ORF102-3	GTCAAGAAAAATAATGGCGG	Just downstream of ORF102-1
ORF100-1	CTATCTCTTTACAGGGACGA	5′ region of truncated *orf100*
ORF100-2	ACACCTTTGCTTGAATCTCA	3′ region of truncated *orf100*
SD1	AGGTGAATTCGTTTGAACGTGCTTTGG	5′ region of *orf101*
ORF101-2	AACGTTCTTCCATTAGTTGA	3′ region of *orf101*
nanE1	TTTCCTGTCAGGCTTTGCCA	5′ region of *nanE*
nanE2	TTCCTTTGGACGTGTGATCG	3′ region of *nanE*
ptsG1	TGTTGCTGGTCTCTTACTGG	5′ region of truncated *ptsG*
ptsG2	CCATACCAGGAATTAGCACGTGAAAT	3′ region of truncated *ptsG*
SD2	CGCAGGATCCAATACAGGAAATTGT	5′ region of truncated *ptsG*
F1	GAGTTTGATCCTGGCTCAG	5′ region of 16S rRNA
R13	AGAAAGGAGGTGATCCAGCC	3′ region of 16S rRNA

tributed. Third, three reference strains of serotypes 20, 22, and 26, which had an atypical type of organization containing only *orf100*, belonged to cluster III, whereas other atypical-type strains of serotypes 13, 21, and 24 were grouped into cluster I, where the strains of serotypes 13 and 21 formed a sister group. And lastly, the 16S rRNA sequence divergence between strain DAT1 (cluster I) and the serotype 7 reference strain (cluster II) was significantly large (distance, 0.0112), although the *orf102* sequences of these two strains were completely identical to each other.

With the exception of atypical-type strains in which the corresponding chromosomal regions have been rearranged, all the

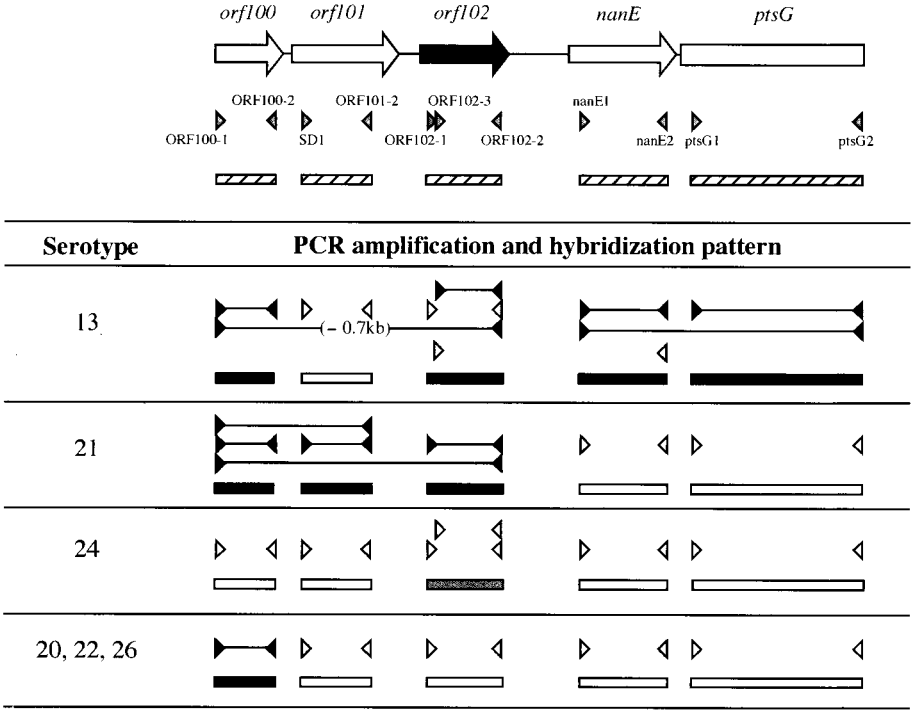

FIG. 3. Schematic representations of the results of systematic PCR and Southern hybridization analyses of atypical-type reference strains. Positions of primers and probes used relative to the DAT1-type genetic organization are indicated at the top by gray arrowheads and hatched boxes, respectively. *orf100*, *orf101*, *orf102*, *nanE*, and *ptsG* probes were amplified by PCR from the genomic DNA of strain DAT1 with primers ORF100-1 and ORF100-2, SD1 and ORF101-2, ORF102-1 and ORF102-2, nanE1 and nanE2, and ptsG1 and ptsG2, respectively. The genomic DNAs of the six reference strains were digested with *Pst*I and *Xho*I. Solid lines between the closed arrowheads, regions amplified by PCR; open arrowheads facing each other, primers that did not amplify any PCR fragment; closed and gray boxes, probes that gave strong and weak hybridization signals, respectively; open boxes, probes that did not give any hybridization signals. When the length of the amplified fragment was different from that expected from the sequence of DAT1, the size difference is indicated in parentheses.

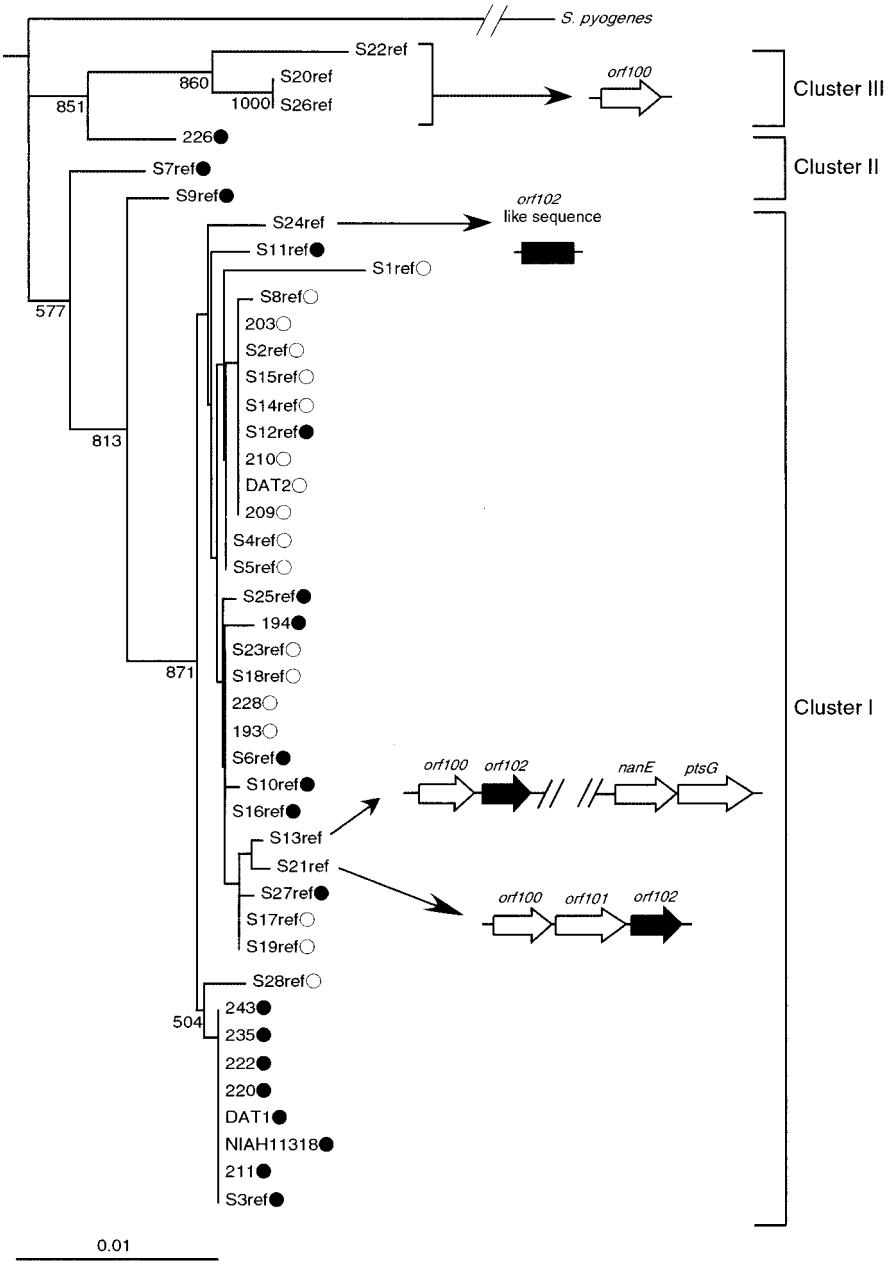

FIG. 4. Distribution of genetic organization types in *S. suis* strains whose positions are shown on a 16S rRNA-based tree constructed by the neighbor-joining method (28). The tree was rooted using the *S. pyogenes* 16S rRNA sequence as the outgroup. The numbers at the nodes of branches indicate the bootstrap values based on 1,000 resamplings. Reference strains of each serotype are referred to by the serotype number sandwiched between "S" and "ref." *S. suis* strains were divided into three clusters on the basis of their distances from the 16S rRNA sequence of the serotype 1 reference strain (NCTC10237 [S1ref]). The distances were calculated by using the DNADIST program (PHYLIP) with the Maximum Likelihood option as the model of nucleotide substitution. Scale bar, sequence dissimilarity; ○, DAT2 type; ●, DAT1 type.

S. suis strains used in this study had either a *sly* or an *orf102* gene at the same location in the genomes between *orf101* and *nanE*, and the *sly*-flanking regions were conserved irrespective of the presence of *sly*. The mutually exclusive localization of *sly* and *orf102* at the same place suggests that at least one of them was horizontally transferred into *S. suis* from a foreign source and was replaced with the gene that had existed between *orf101* and *nanE*. Although natural transformation has not yet been demonstrated for *S. suis*, the presence of at least two genes which showed homology to competence-related genes has been indicated (33) and we have occasionally found several competence-related genes from shotgun sample sequencing data of the *S. suis* NCTC10234 genomic library (unpublished observations). Therefore, it is plausible that the *sly* or *orf102* gene may be delivered into a recipient *S. suis* strain via a transformation event. No vestiges of the sequences affecting their integration, such as long-repeat DNA sequences or remnants of translocatable elements, were found in the vicinity of

TABLE 3. Nucleotide sequence accession numbers

Gene or region	*S. suis* strain[a]	Accession no.
16S rRNA	DAT1	AB071336
	DAT2	AB071337
	NIAH11318	AB071338
	193	AB071339
	194	AB071340
	203	AB071341
	209	AB071342
	210	AB071343
	211	AB071344
	220	AB071345
	222	AB071346
	226	AB071347
	228	AB071348
	235	AB071349
	243	AB071350
sly region	DAT2	AB055649
sly	203	AB071351
	NCTC10237 (serotype 1)	AB071353
	6407 (serotype 4)	AB071354
	14636 (serotype 8)	AB071355
	42A (serotype 19)	AB071356
	89-2479 (serotype 23)	AB071357
	89-590 (serotype 28)	AB071358
orf102 region	DAT1	AB071359
orf102	226	AB071360
	2524 (serotype 6)	AB071361
	8074 (serotype 7)	AB071362
	22083 (serotype 9)	AB071363
	8830 (serotype 12)	AB071364
nanE with upstream region	10581 (serotype 13)	AB071365

[a] Only the serotypes of the reference strains are indicated.

sly and *orf102*, while a BOX-like element was found downstream of the *orf102* in DAT1 (Fig. 2). Unique structures found in the flanking region, which showed relatively low homology (Fig. 1 and 2), may suggest that the original incorporation of *sly* or *orf102* into the *S. suis* genome has occurred via a unique mechanism of gene transfer rather than by the insertion of a mobile genetic element. Phylogenetic analysis suggests that the *sly* and the *orf102* gene regions were also transferred among *S. suis* strains, and hence the incorporation of DNA was apparently mediated by homologous recombination via conserved flanking regions. Alternatively, the results, especially those obtained for strain DAT1 and the reference strain of serotype 7, raise the possibility that the 16S rRNA gene region could also be transferred among the strains.

An R-M system can work as a barrier to the incorporation of foreign DNA; however, the genetic exchange could occur within an appropriate combination of the strains, i.e., between strains carrying the same R-M system and between strains lacking an R-M system or from the former to the latter. Therefore, our findings about the genetic structures of *sly* loci and their distribution in the *S. suis* population, together with the findings of previous reports (31, 32), suggest that a series of gene transfers, in which a foreign gene is acquired by a certain mechanism and subsequently spread among the strains, is a common occurrence in *S. suis* and that such genomic conversions may contribute to the heterogeneity of the population.

Nucleotide sequence accession numbers. The nucleotide sequences determined in this study have been deposited in the DDBJ, EMBL, and GenBank databases, and their accession numbers are listed in Table 3.

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