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Molecular prophage typing of *Staphylococcus aureus* isolates from bovine mastitis

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Staphylococcus aureus is one of the major pathogens causing bovine mastitis and foodborne diseases associated with dairy products. To determine the genetic relationships between human and bovine or bovine isolates of *S. aureus*, various molecular methods have been used. Previously we developed an *rpoB* sequence typing (RSTing) method for molecular differentiation of *S. aureus* isolates and identification of *RpoB*-related antibiotic resistance. In this study, we performed *spa* typing and RSTing with 84 isolates from mastitic cows (22 farms, 72 cows, and 84 udders) and developed a molecular prophage typing (mPPTing) method for molecular epidemiological analysis of bovine mastitis. To compare the results, human isolates from patients (n = 14) and GenBank (n = 166) were used for real and *in silico* RSTing and mPPTing, respectively. Based on the results, RST10-2 and RST4-1 were the most common *rpoB* sequence types (RSTs) in cows and humans, respectively, and most isolates from cows and humans clearly differed. Antibiotic resistance-related RSTs were not detected in the cow isolates. A single dominant prophage type and gradual evolution through prophage acquisition were apparent in most of the tested farms. Thus, RSTing and mPPTing are informative, simple, and economic methods for molecular epidemiological analysis of *S. aureus* infections.

Keywords: *Staphylococcus aureus*, bovine mastitis, molecular epidemiology, molecular prophage typing, *rpoB* sequence typing

Introduction

Staphylococcus aureus causes bovine mastitis and food poisoning and is an important pathogen in the dairy industry [25,40]. Since 1961, methicillin-resistant *S. aureus* (MRSA) has become an important bacterial threat worldwide. Molecular epidemiological investigations have been performed using various isolates of *S. aureus* from humans and animals [15,42]. Although comparative genomics has been successfully applied to elucidate the transmission and evolution of whole genetic contents related to antibiotic resistance and pathogenicity, it remains costly and time-consuming when analyzing large datasets. Sub-genomic methods including multi-locus sequence typing (MLST), arbitrary primer-polymerase chain reaction (PCR), pulsed-field gel electrophoresis, multi-locus variable number tandem repeat analysis, *spa* typing, small genomic islet typing, *rpoB* sequence typing (RSTing), and microarray hybridization have been used [5,8,11,26-28,33,41]. Among these methods, RSTing has been developed to reduce the experimental cost of MLST and was successfully applied to the molecular epidemiological study of human and avian isolates of *S. aureus* [33]. Furthermore, RSTing provides information on rifampin, daptomycin, and vancomycin resistance in *S. aureus*, which are useful for MRSA treatment [1,6,45,46].

Prophage typing after induction with mitomycin C has been used to characterize *S. aureus* strains [21]. Temperate bacteriophages (phages) carry and mobilize diverse virulence genes, and prophage profiles may help predict the potential pathogenicity of *S. aureus* isolates [2,14,20,29,44]. The terminase large subunit gene was used to identify prophages in bacterial genomes and for the differentiation of phages from environments and uncultured bacteria [3,4,35]. A molecular prophage typing (mPPTing) method based on the terminase large subunit gene has been developed for molecular epidemiological study of avian pathogenic *Escherichia coli* in chickens and has provided additional information regarding

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phage-carrying virulence genes [24]. In addition, mPPTing with integrase genes in temperate phages of *S. aureus* has been developed [9].

In the present study, we developed a novel mPPTing method by using terminase large subunit genes for 84 and 14 isolates of *S. aureus* from bovine mastitis and humans, respectively, and compared the results with those from *in silico* mPPTing of 166 human *S. aureus* strains in the GenBank database. This mPPTing method provides information about prophage profiles carrying virulence genes as well as on the molecular epidemiology of *S. aureus* infection.

Materials and Methods

Bacterial isolates

A total of 84 *S. aureus* isolates from bovine mastitis cows (22 farms, 72 cows, and 84 udders) in Korea were isolated after cultivation on sheep blood agar and identified by using the VITEK2 system and Gram-Positive Identification Cards (BioMérieux, France) [18]. Fourteen human MRSA isolates were purchased from the Culture Collection of Antimicrobial Resistant Microbes (Korea), and MLST and *rpoB* sequence type (RST) were characterized previously [33] (Supplementary Table 1). The 166 human strains were collected from the genome sequences of *S. aureus* in the GenBank database (Supplementary Table 1).

Primers

The primer sets for RSTing and detection of the methicillin-resistance gene, *mecA*, and *spa* typing were used as previously reported [19,33,38]. The primer sets for mPPTing were designed by using large subunit terminase genes of *S. aureus* collected from the GenBank database; primer sequences are summarized in Table 1. Primer sets for detecting prophage-related virulence genes, as well as staphylokinase (*sak*) and chemotaxis-inhibiting (*cip*) genes, were designed and used in this study (sakF, 5′-GCGAT GACGC GAGTT ATTTT-3′ [116–135]; sakR, 5′-GACTT CGATC TTTGC GCTTG-3′ [329–348]; cipF, 5′-GCAGG AATCA GTACA CACCA TCATT CAG-3′ [49–76]; cipR, 5′-CAGCA AGTGG TGTAT TCAGA TATAC TGTAT AG-3′ [297–328]).

PCR and sequencing

The *S. aureus* strains were grown overnight in tryptic soy broth at 37°C, and genomic DNA was extracted from 1 mL of *S. aureus* tryptic soy broth culture by using a G-spin For Bacteria

Table 1. Primer sets for polymerase chain reaction and sequencing of terminase large subunit genes

Prophage group	Primer				Homologous Phage			Frequency (%)	
	Name	Sequence (5′–3′)	Location	Amplicon size (bp)	Name	Accession No.	Genome size (bp)	Bovine (n = 84)	Human* (n = 180)
1	1F	CCATCTATTTTACGATATCTGC	107–128	1585	SMSAP5 (Sipho)	JQ779023	45552	40.5	51.7
	1R	GTGACTATTAAAGTTTTAAATG	1671–1692						
2	2F	CATATTCGAAATACTAACCA	54–73	1212	NM2 (Sipho)	DQ530360	43135	23.8	28.3
	2R	GATTTTATTGTGTCAACTTTCG	1244–1265						
3	3F	TATTTTGGACGAATTGGCAAG	591–611	813	13 (Sipho)	AF424783	42722	21.4	13.8
	3R	GGCCAATCTAAAGCCATTGA	1384–1403						
5	5F	ATTTGGCGTTCAAGTGGTTC	306–325	645	StauST398-3 (Sipho)	JQ973847	41392	0	10.2
	5R	GGTCGTGCAGTATCGCAGTA	931–950						
6	6F	AGGGGGCTTGGTAAGACATT	250–269	575	SPbeta-like (Sipho)	KT429160	127726	0	3.9
	6R	TTGAGCTTCGGCTTTCATTT	824–843						
7	7F	ACGCCAAAAGCTCCTTATGA	184–203	895	P282 (Sipho)	KT809368	41960	26.2	69.4
	7R	CCTCAAACGCACGTCTTACA	1059–1078						
8	8F	GTATTAATAGCGAGTGGTGC	70–89	1116	SA13 (Sipho)	JX094501	42652	11.9	9.4
	8R	CTTATTAGCTGTATAAACTGC	1165–1185						
9	9F	TGATGAACAGAAAATCGAGGA	132–152	1223	P282 (Sipho)	KT809368	41960	11.9	69.4
	9R	CCTCAAACGCACGTCTTACA	1335–1354						
10	10F	GATCAGTATCAATCAACAATGG	433–454	1769	SA13 (Sipho)	JX094501	42652	17.9	8.9
	10R	GATTGCATGTATTGCCAAATCG	2180–2201						
12	12F	GGATACTCATTTTATGGCAAAG	3–24	2211	ETA3 (Sipho)	AP008954	43282	0	4.2
	12R	GTGATTGTACTCCATATCTG	2194–2213						

F, forward; R, reverse. **In silico* analysis of *Staphylococcus aureus* strains with complete genome sequences.

Genomic DNA Extraction Kit (iNtRON Biotechnology, Korea) according to the manufacturer's instructions. The *rpoB* PCR mixture was composed of 5 μL of 10× reaction buffer, dNTPs (10 mM, 2.5 μL), Exprime Taq DNA polymerase (5 U/μL; Genet Bio, Korea; 0.5 μL), forward and reverse primers (1. 25 μM, 1.25 μL of each), distilled water (38.5 μL), and template DNA (50 ng/μL, 1 μL). The mixture was incubated at 94°C for 5 min; 35 cycles of 94°C for 30 sec–54°C for 30 sec–72°C for 4 min with a final extension step at 72°C for 5 min. The PCR for *spa* typing was performed as described for *rpoB* PCR except for the PCR condition: 80°C for 5 min; 35 cycles at 94°C for 45 sec–60°C for 45 sec–72°C for 90 sec with a final extension step at 72°C for 10 min [38]. The PCR for mPPTing and for *sak* and *cip* were performed as described for *rpoB* PCR except for elongation times of 2 min and 30 sec, respectively, were used for the 35 cycles. PCR for *mecA* was performed as described previously [19].

The PCR amplicons were purified using the MEGAquick-

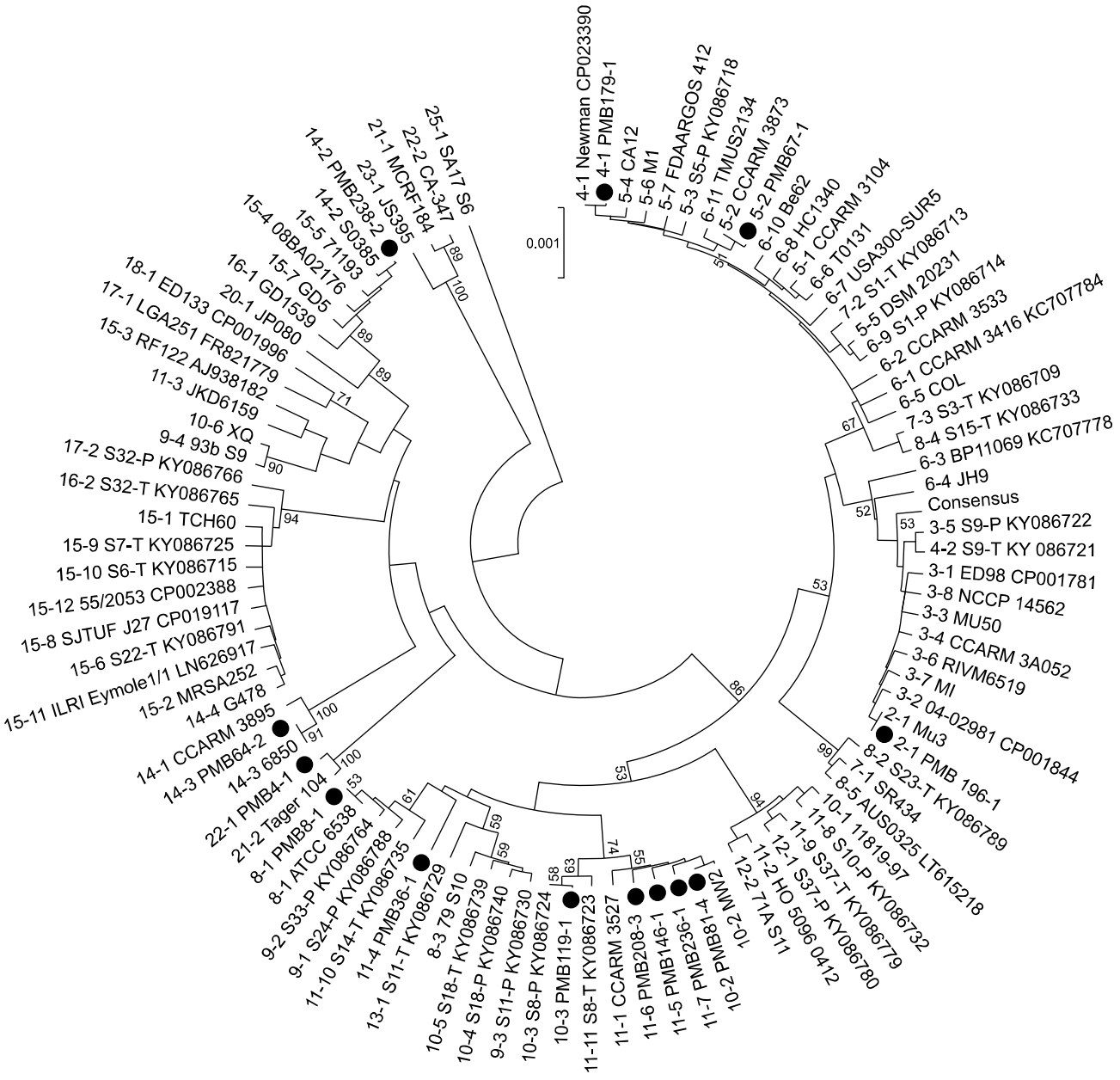

Fig. 1. Phylogenetic analysis of the complete *rpoB* sequences of 90 representative *rpoB* sequence types and consensus sequence of *Staphylococcus aureus*. The phylogenetic tree was constructed by using the neighbor-joining method (p-distance and 1,000 bootstrapping replicates) with MEGA software (ver. 7) [23]. The bovine isolates analyzed in this study are marked with closed circles.

spin Total Fragment DNA Purification Kit (iNtRON Biotechnology) and sequenced with sequencing primers using an ABI3711 automatic sequencer (Macrogen, Korea).

Sequence analysis

Overlapping sequences of the *rpoB* genes of *S. aureus* isolates from bovine mastitis were assembled into single complete sequences by using ChromasPro (ver. 1.5; Technelysium, Australia). After comparison with consensus and previously identified RST sequences, the RST of the new *rpoB* sequence was assigned as previously described [33]. Nucleotide similarity, variable nucleotide comparisons, and translation of nucleotide sequences were performed with Bioedit software (ver. 5.0.9.1; Ibis Biosciences, USA). Phylogenetic analyses with complete *rpoB* genes were conducted using MEGA software (ver. 7; neighbor-joining method with p-distance and 1,000 repeats of bootstrapping) [23]. The *spa* type of each *S. aureus* isolate was determined by using BioNumerics (ver. 7.6; Applied Math, Belgium).

In silico mPPTing was performed by querying the representative sequence of each prophage group by BLAST searching and confirming 100% nucleotide matches of the primer regions. *In silico* MLST was performed by querying the retrieved housekeeping genes of reference strains in which the sequence type (ST) was not described in the GenBank files by using BLAST based on the allele templates in the MLST database.

Statistical analysis

The index of diversity (ID) and confidence interval (CI, 95%) were calculated as previously described [10]. The frequencies of RSTs, *spa* types, phage groups, and molecular prophage types (mPPTs) were compared via chi-squared and Fisher's exact tests (95% CI) using SPSS for Windows (ver. 12.0; SPSS, USA).

Results

Complete *rpoB* gene sequences of *S. aureus* isolates from bovine mastitis

The complete *rpoB* coding region of *S. aureus* composed of 3,552 nucleotides was successfully amplified by the PCR primer set. The complete *rpoB* sequences of the bovine *S. aureus* isolates in this study were deposited in GenBank (MG737590–MG737673) and the accession numbers of all *rpoB* sequences used for analysis are separately shown in Fig. 1 and Supplementary Table 1. Nucleotide similarities between *rpoB* sequences were 99.0% to 100%, and the amino acid changes from the consensus sequence are summarized in Table 2. Amino acid changes related to rifampin, daptomycin, and vancomycin resistance are also summarized in Table 2. No bovine *S. aureus* isolates showed amino acid changes related to rifampin, daptomycin, and vancomycin resistance [1,45,46]. Therefore, they may have no or low resistance to those antibiotics.

Actual and *in silico* RSTing of *S. aureus* from cows and humans

The complete *rpoB* sequences of 84 bovine isolates were compared with the consensus sequence and classified into 13 RSTs as previously described [33] (Table 3). We collected all *rpoB* sequences from human, animals, and the environment in the GenBank database and classified these sequences into RSTs. A total of 89 RSTs from RST2-1 to RST25-1 were assigned to all *S. aureus* isolates and strains (Table 4). The representative *rpoB* sequence of each RST was used for

Table 2. Amino acid changes of RpoB in *rpoB* sequence types (RSTs) of *Staphylococcus aureus*

Function	Mutation (RST)	Reference
RRDR*; unknown	S463N (6-1), D471N (6-10), E538K (15-4)	
RRDR; Rif†	S464P (6-1), L466S (6-6, 7-3, 8-4), H481N (5-1, 6-6, 6-8, 6-10, 7-3, 8-4, 11-10, 12-1, 15-7), H481Y (3-3, 6-7, 6-11)	[1,39]
RRDR; Rif† VISA‡	D471Y/ A473S/A477S/ E478D (6-4)	[1,38,39]
VISA	Y737F (14-4, 15-1, 15-2, 15-6, 15-8, 15-9, 15-10, 15-11, 15-12, 16-2, 17-2)	[38]
Rifampin sensitive	V135L (6-8), R140S (3-7), D320N (21-1, 22-2, 23-1)	[38]
Unknown	E43K (6-3), V126I (22-1), G129D (3-8) Q137R (6-7), A160S (3-5, 4-2), D185Y (5-7), T193A (11-5), D209G (8-5), T229A (16-1), N276T (16-2, 17-2), L279F (17-2), H283Q (17-1), T308R (15-11), D355E (17-1, 18-1), V362A (16-1), T518M (15-9), T553I (11-1), A576V (5-4), D631E (13-1), D707N (10-6), V731A (15-1), A798V (6-5), N822D (9-3, 10-4, 10-5, 13-1), S875L (6-5), R917S (4-2), P946S (15-10), P990S (11-8), E1006K (6-2), D1046V (11-12), K1133E (3-4), G1139V (9-2, 11-11), R1165H (3-1), T1182I (5-3, 7-2, 7-3, 8-2, 9-1, 10-5, 11-9, 11-11, 13-1, 15-6, 17-2)	

*Rifampin resistance determining region. †Rifampin resistance. ‡Vancomycin-intermediate *S. aureus*.

Table 3. *RpoB* sequence typing (RSTing) and molecular prophage typing (mPPTing) of *Staphylococcus aureus* isolates from bovine mastitis (22 farms, 72 cows and 84 udders)

RSTing		mPPTing		Strain
RST	Frequency	mPPT	Frequency	
10-2	6(27.3)-29(37.7)-35(41.7)*	0	3-9-10	PMB64-1(t2459), PMB132-1-1(t189), PMB132-1-2(t189), PMB132-2(t189), PMB132-3(t189), PMB132-4(t189), PMB132-6(t189), PMB132-7(t189), PMB232-2(t127), PMB232-3(t127)
		1	1-1-1	PMB132-5(t189)
		3	1-1-1	PMB232-1(t127)
		8	1-1-1	PMB46-1(t2612)
		10	1-1-2	PMB61-1-2(t189), PMB61-1-3(t189)
		1-2	1-1-1	PMB81-11(t127)
		1-3	1-1-1	PMB81-8(t127)
		2-10	1-1-1	PMB2-1†(t189)
		8-10	1-1-1	PMB61-1-1(t189)
		1-2-3	1-13-16	PMB81-1(t127), PMB81-2(t127), PMB81-3-1(t127), PMB81-3-2(t127), PMB81-4(t127), PMB81-5-1(t127), PMB81-5-2(t127), PMB81-6(t127), PMB81-7-1(t127), PMB81-7-2(t127), PMB81-9(t127), PMB81-10(t127), PMB81-12(t127), PMB81-13(t127), PMB81-14(t127), PMB81-15(t127)
10-3	3(13.6)-8(10.4)-8(9.5)*	0	1-1-1	PMB173-1(UT1)
		7	1-1-1	PMB188-1(UT2)
		10	1-6-6	PMB119-1(t304), PMB119-2(t304), PMB119-3(t304), PMB119-4(t304), PMB119-5(t304), PMB119-6(t304)
14-2	2-6-6	1-7-9	2-6-6	PMB177-1(t034), PMB177-2(t034), PMB238-1(t034), PMB238-2(t034), PMB238-3(t034), PMB238-4(t034)
11-4	2-5-5	10	1-1-1	PMB242-4(t164)
		8-10	1-1-3	PMB242-1(t164), PMB242-2(t164), PMB242-3(t164)
		7-8-9-10	1-1-1	PMB36-1(t164)
14-3	2-2-2	1	2-2-2	PMB64-2(t2459), PMB66-1(t002)
22-1	2-2-2	1-2	2-2-2	PMB4-1(t4050), PMB5-1‡(t4050)
2-1	1-9-11	7	1-5-7	PMB196-1(t002), PMB196-2-1(t002), PMB196-2-2(t002), PMB196-3-1(t002), PMB196-3-2(t002), PMB196-5-2(t002), PMB196-6(t002)
		7-8	1-4-4	PMB196-4(t002), PMB196-5-1(t002), PMB196-7(t002), PMB196-8(t002)
4-1	1-4-7	1	1-4-7	PMB179-1(t084), PMB179-2-1(t084), PMB179-2-2(t084), PMB179-2-3(t084), PMB179-3-1(t084), PMB179-3-2(t084), PMB179-4(t084)
11-6	1-3-3	0	1-1-1	PMB208-3(t189)
		7-9	1-2-2	PMB208-1(t189), PMB208-2(t189)
8-1	1-2-2	0	1-2-2	PMB8-1(UT3), PMB8-2(UT4)
5-2	1-1-1	7-9	1-1-1	PMB67-1(t002)
11-5	1-1-1	0	1-1-1	PMB146-1(t189)
11-7	1-1-1	0	1-1-1	PMB236-1(t127)

UT, untypable variant. *Number of farm (%)-number of cow (%)-number of udder (%). †PMB2 and PMB61 were isolated from the same farm on different dates. ‡PMB5 and PMB242 were isolated from the same farm at different dates.

phylogenetic analysis and these sequences formed distinct clusters with each other in the phylogenetic tree (Fig. 1). Particularly, RSTs with neighboring numbers, such as 2–3, 4–7, 8–11, 14–17, and 21–23, formed a distinct cluster with other RSTs. The frequencies of RSTs of bovine isolates and human strains of *S. aureus* are summarized in Table 3. RSTs 10-2 (41.7%) and 4-1 (37.1%) were the most common among bovine isolates and human strains, respectively, and accounted for

Table 4. Real and *in silico* molecular typing of *rpoB* sequence types (RSTs) and prophage types (PPTs) of *Staphylococcus aureus* strains in GenBank (n = 180)

RSTing		mPPTing		RSTing		mPPTing	
RST	Frequency	mPPT	Frequency	RST	Frequency	mPPT	Frequency
2-1	24 (13.3%)	2	3	6-2	1	6-7-8-9-10	1
		3	1	6-4	1	1-7-8-9-10	1
		7-9	11	6-5	1	1	1
		1-7-9	2	6-6	1	7-9	1
		2-7-9	2	6-7	6	1-2-7-9	6
		5-7-9	1	6-8	2	1-5-7-9-12	2
		1-2-7-9	1	6-10	1	1-2-7-9	1
		1-2-8-10	1	6-11	1	7-9	1
		7-8-9-10	1	7-1	2	7-9	1
		1-7-8-9-10	1			5-7-9	1
3-2	1	7-9	1	8-1	1	3	1
3-4	1	2-8-10	1	8-3	3	7-9	2
3-3	1	2-7-9	1			2-7-9	1
3-6	1	7-8-9	1	9-4	1	1-2	1
3-7	1	7-9	1	10-1	4	1-3	2
3-8	1	1-8-10	1			7-9	1
4-1	64 (35.6%)	0	3			1-2-3	1
		1	1	10-2	6	0	1
		3	3			1-3	5
		7-9	1	11-1	2	7-9	2
		1-3	1	11-2	2	1-7-9	2
		5-10	2	11-3	1	1-7-9	1
		1-2-3	2	12-2	1	3-7-9	1
		1-5-12	1	14-1	7	0	5
		1-7-9	21			7-9	1
		2-7-9	1			5-7-9	1
		7-8-9	1	14-2	7	0	1
		1-2-7-9	20			1	3
		2-5-7-9	1			1-5	1
		3-5-6-12	1			7-9	1
		1-5-7-9-12	1			1-7-9	1
		6-7-8-9-10	3	14-3	1	0	1
		5-6-7-8-9-10-12	1	14-4	3	3	3
5-1	8	1-2-7-9	2	15-1	1	1	1
		1-5-7-9	1	15-2	1	1-7-9	1
		1-7-8-9	1	15-4	1	1	1
		7-8-9-10	3	15-5	1	7-9	1
		5-6-7-9-12	1	15-7	1	7-9	1
5-2	7	0	1	16-1	1	1-5-7-9	1
		7-9	5	20-1	1	3	1
		2-7-9	1	21-1	1	0	1
5-4	1	1-7-9	1	21-2	1	2-7-9	1
5-5	1	0	1	22-2	1	1-5-7-9	1
5-6	1	1-3	1	23-1	1	0	1
5-7	1	7-9	1	25-1	1	1-7-9	1

51.2% and 48.9% of isolates, respectively, to sum up the first and the second most predominant genotypes of bovine isolates (RST10-3) and human strains (RST2-1). The RSTs 2-1, 4-1, 5-2, 8-1, 10-2, 14-2, and 14-3 were commonly present in bovine isolates and human strains, while RSTs 10-3, 11-4, 11-5, 11-6, 11-7, and 22-1 were only detected in bovine isolates. The frequency of RST10-2 in bovine isolates (41.7%, 35/84) was significantly higher than that (2.8%, 5/180) in human isolates of *S. aureus* ($p < 0.05$).

The discriminative power of RSTing and MLST was compared with those of actual and *in silico* results presented in Supplementary Table 1. The RSTs 2-1, 4-1, 7-1, 10-1, 10-2, and 14-2 were composed of 4, 8, 2, 2, 2, and 2 different STs, respectively. The 5, 8, 239, 72, 22, 1, 398, and 45 STs were composed of 6, 5, 6, 2, 2, 2, 5, and 2 different RSTs. Thus, RSTing could not differentiate 20 STs, while MLST could not differentiate 30 RSTs. However, the IDs (CIs) of RSTing and MLST were 85.1% (80.6–89.5%) and 86.7% (82.6–90.8%), respectively, when they were calculated on the basis of data in Supplementary Table 1, except for the not tested and untypable (UT) strains. Therefore, RSTing showed slightly less discriminative power than MLST.

spa typing of *S. aureus* isolates from bovine mastitis

The *spa* types of 84 bovine isolates were determined (Table 3). Eighty isolates were assigned into 10 *spa* types, t127 (26.2%, 22/84), t189 (19.0%, 16/84), t002 (15.5%, 13/84), t084 (8.3%, 7/84), t034 (7.1%, 6/84), t304 (7.1%, 6/84), t164 (6.0%, 5/84), t2459 (2.4%, 2/84), t4050 (2.4%, 2/84), t2612 (1.2%, 1/84). Four isolates were classified into 4 UT variants, PMB 173-1 (11-10-21-17-172-24-34-22-25, UT1), PMB 188-1 (11-19-17-34-22-25-25-25, UT2), PMB 8-1 (26-17-34-34-34-34-33-34, UT3), PMB 8-2 (26-17-34-34-34-34-22-34-34-34-34-33-34, UT4). Most of the isolates from the same farm showed the same *spa* type and the farm-based frequency of each *spa* type was as follows: 13.6% (3/22; t127, t189, and t002), 9.1% (2/22; t034, t164, and t4050), and 4.5% (1/22; t084, t304, t2612, t2459, UT1, UT2, UT3, and UT4). The major RST10-2 was composed of 4 *spa* types including two major *spa* types, t127 and t189. Relatively high frequencies of t127 and t189 among *S. aureus* isolates of bovine mastitis have already been reported in Korea and Brazil, and South Africa, respectively [13,32,36].

Amplification of terminase large subunit gene of *S. aureus* isolates from bovine mastitis and human patients

The terminase large subunit genes of prophages were collected from the GenBank database by keyword and BLAST searches and then subjected to phylogenetic analysis. Terminase genes forming a cluster were aligned and a cluster-specific PCR primer set was designed (Table 1). The PCR results are shown in Fig. 2. Arabic numbers were assigned to prophage groups (1, 2, 3, 5, 6, 7, 8, 9, 10, and 12), and a representative phage from each prophage group with high nucleotide identity (not less than 99%) is summarized with its name, accession number, and genome size in Table 1. All representative phages were classified into Family *Siphoviridae*, and φNM2, φ13, φSPβ-like, and φETA3 have been reported to possess virulence genes [2,14,20]. Eighty-four and 14 cow isolates and human strains, respectively, were tested for the presence of each prophage group. Prophage group 1 (40.5%) was the most frequent in bovine isolates, followed by phage groups 7 (26.2%), 2 (23.8%), 3 (21.4%), 10 (17.9%), 8 (11.9%), and 9 (11.9%). Phage groups 5, 6, and 12 were not detected. The frequency of phage groups of *S. aureus* isolates from human patients were combined with *in silico* results that were predicted by using genome sequences of human *S. aureus* strains in the GenBank database. According to the results, phage groups 7 and 9 (69.4%) were the most frequent, followed by phage groups 1 (55.1%), 2 (25.7%), 3 (13.8%), 5 (10.2%), 8 (7.2%), 10 (6.6%), 12 (4.2%), and 6 (3.0%). The frequencies of phage groups 7 and 9 in human isolates were significantly higher than those in bovine isolates of *S. aureus* ($p < 0.05$).

Actual and *in silico* mPPTing of *S. aureus* from cows and humans

The real and *in silico* mPPTs of each *S. aureus* isolate are summarized in Table 4 and Supplementary Table 1. The mPPT is represented as a hyphen-separated number of prophage groups possessed by a given isolate with mPPT0 assigned to isolates without a detected prophage group. Each RST was divided into subgroups of mPPTs, and *S. aureus* isolates from the same farm or the same individual showed the same or similar mPPTs. Interestingly, the genotype, RST10-2/mPPT0

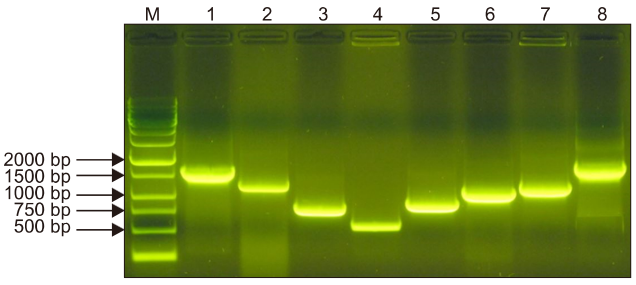

Fig. 2. Amplification of terminase large subunit genes of prophage groups 1, 2, 3, 6, 7, 8, 9, and 10. Amplicons of prophage groups 5 and 12 are not shown because positive bovine isolates and human strains were not available. Lane M, 1000 bp size marker; Lane 1, prophage group 1 (1585 bp); Lane 2, prophage group 2 (1212 bp); Lane 3, prophage group 3 (813 bp); Lane 4, prophage group 6 (575 bp); Lane 5, prophage group 7 (895 bp); Lane 6, prophage group 8 (1116 bp); Lane 7, prophage group 9 (1223 bp); Lane 8, prophage group 10 (1769 bp).

was dominant compared to other genotypes and was distributed on a limited number of dairy farms (4/26 farms, 15.4%). The frequency of mPPT0 among different RSTs was higher in bovine isolates (6/13 RSTs, 46.2%) than in human strains (10/49 RSTs, 20.4%), but the difference was not significant. In addition, mPPT7-9 (31/180, 17.2%) was the most frequent in human strains, followed by mPPTs 1-7-9 (30/180, 16.7%) and 1-2-7-9 (30/180, 16.7%). Step-wise, cumulative acquisition of the prophage was apparent in RST10-2 and RST11-4, and RST2-1 and RST4-1 of bovine isolates and human strains, respectively. The mPPTs possessing at least 5 prophage groups were absent in bovine isolates but present in the 2-1, 4-1, 5-1, 6-2, 6-4, and 6-8 RSTs of human strains.

Bovine isolates with the same RSTs/mPPTs as human strains were present. RST10-2/mPPT0, RST10-2/mPPT1-3, RST14-2/mPPT1-7-9, and RST5-2/mPPT7-9 were also present in human strains, and multiple strains of RST10-2/mPPT1-3 and RST5-2/mPPT7-9 were present in 5 and 3 strains, respectively.

Comparison of discriminatory powers of *spa* typing, RSTing, and mPPTing

We calculated IDs and CIs of *spa* typing, RSTing, and mPPTing for 84 bovine isolates of *S. aureus*. The IDs (Cis) of *spa* typing, RSTing, and mPPTing were 85.9% (82.2–89.6%), 79.1% (71.7–86.5%), and 90.3% (87.8–92.8%), respectively. Thus, *spa* typing had higher discriminative power than RSTing but less than that of mPPTing.

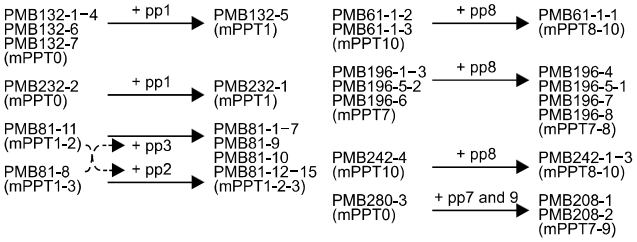

Fig. 3. Evolution of *Staphylococcus aureus* on dairy farms through the acquisition of temperate phages. The first, second, and third numbers of each isolate's name are identifiers of the farm, individual cow, and udder, respectively. mPPT, molecular prophage type; + pp, acquisition of prophage.

Transmission and molecular evolution of *S. aureus* on dairy farms

In most cases, *S. aureus* isolates from a farm had the same RSTs and the same or slightly different mPPTs. Of the 13 multi-sampled dairy farms, a single genotype of *S. aureus* was observed on 5 farms (PMB8, PMB119, PMB177, PMB179, and PMB238; 38.5%) and mixed genotypes composed of parent and progeny genotypes of *S. aureus* were observed on 7 farms (PMB61, PMB81, PMB132, PMB196, PMB208, PMB232, and PMB242; 53.8%). Evolution of the dominant genotype via acquiring new prophages on dairy farms of mixed genotypes was also apparent (Fig. 3). The mPPT10 (PMB61-1-1, PMB61-1-2, and PMB242-4) and mPPT7 (PMB196-1, 2, 3, 5-2, and 6) acquired a prophage group 8 phage to become mPPT8-10 (PMB61-1-3, PMB242-1, 2, and 3) and mPPT7-8 (PMB196-4, 5-1, 7, and 8), respectively. The mPPT0 (PMB231-2; PMB132-1, 2, 3, 4, 6, and 7; PMB208-3) became mPPT1 (PMB232-1 and PMB132-5) and mPPT7-9 (PMB208-1 and 2) by acquiring prophage group 1 and 7-9 phages, respectively. In addition, mPPT1-2 (PMB81-11) and mPPT1-3 (PMB81-8) became mPPT1-2-3 by acquiring prophage groups 2 and 3 phages, respectively. Therefore, transmission of the dominant genotype of *S. aureus* between individual cows and evolution by the acquisition of prophages on dairy farms was commonly observed.

Detection of methicillin-resistance and virulence genes

MecA was not detected in any isolate and might be infrequent in Korean bovine isolates. The representative phages of prophage groups 3 and 7 are φ13 and φP282, respectively.

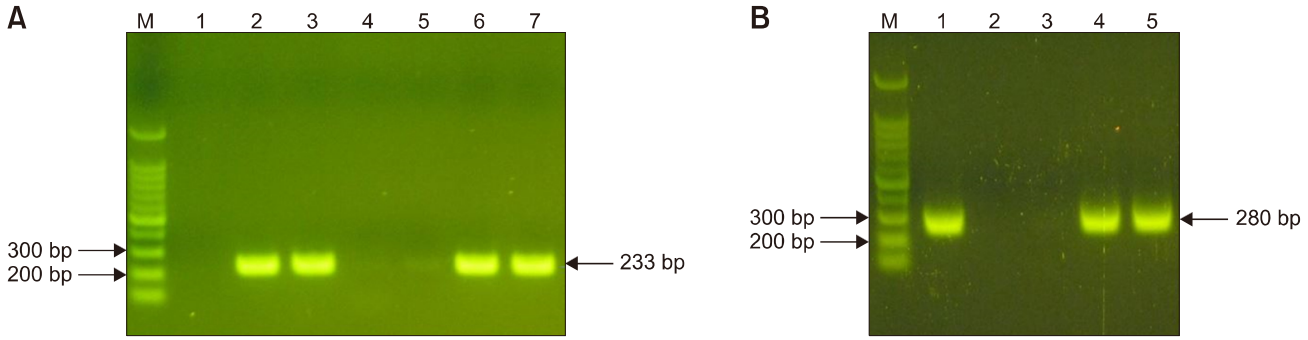

Fig. 4. Virulence genes of prophage groups 3 and 7. The staphylokinase (A) and chemotaxis inhibiting (B) genes were detected by polymerase chain reaction. (A) Lane M, 100 bp size marker; Lane 1, PMB 81-1; Lane 2, PMB 232-1; Lane 3, PMB 67-1; Lane 4, PMB 177-1; Lane 5, PMB 188-1; Lane 6, PMB 196-1; Lane 7, PMB 208-1. (B) Lane M, 100 bp size marker; Lane 1, PMB 67-1; Lane 2, PMB 177-1; Lane 3, PMB 188-1; Lane 4, PMB 196-1; Lane 5, PMB 208-1.

Phages φ13 (*sak*) and φP282 (*sak* and *cip*) carry virulence genes; thus, we tested the presence of the virulence genes in the bovine isolates. Of prophage group 3-positive isolates (PMB81-1 and PMB232-1), PMB232-1 possessed *sak,* and of prophage group 7-positive isolates (PMB67-1, PMB177-1, PMB188-1, PMB196-1, and PMB208-1), PMB67-1, PMB196-1, and PMB208-1 contained *sak* and *cip* (Fig. 4).

Discussion

Various sub-genomic molecular typing methods have been successfully applied to *S. aureus* isolates, and such methods can provide information regarding genetic diversity for use in molecular epidemiological studies. Among these methods, MLST has been widely applied to various pathogenic bacteria, but its handling of multiple genes, low discriminative power at the serotype level, and lack of clinical information for the data limit the applicability of this method. Thus, we developed the RSTing method for molecular serotyping of *Salmonella enterica* subsp. *enterica* and examining the molecular epidemiology of *S. aureus* isolates [33,34].

In this study, we used RSTing to investigate the molecular epidemiology of bovine mastitis caused by *S. aureus* isolates. The predominance of certain genotypes of *S. aureus* in different host species has been reported, which was confirmed by the results of the RSTing method [7,17,33,39]. RSTs 10-2 and 10-3, and 4-1, and 2-1 were predominant, accounting for approximately 50% of bovine isolates and human strains. The greater diversity among the RSTs of human strains (48) compared to that of bovine isolates (13) supports the observation that *S. aureus* of humans spread to animals and has adapted to specific niches in animals [30,31]. The presence of RSTs 2-1 and 4-1 in bovine isolates may reflect human to animal transmission, but transmission by contact with humans may occur occasionally based on the relatively low frequencies of the RSTs. However, transmissions of animal-specific *S. aureus* to humans was commonly observed and livestock-associated MRSA (LA-MRSA) has been reported [12,43]. RST10-2 was observed in human strains, and FORC 026 and FORC 045 strains isolated from Korean patients completely matched the Korean bovine isolates' mPPTs. These strains may be transmitted occasionally by foodborne diseases. Therefore, recent, not infrequent transmission of LA-MRSA from pigs and cows to humans may be a reason for the application of RSTing and mPPTing methods to unravel epidemiological relatedness between them [12,37].

The first RST identifier number represents the number of nucleotides mismatched to the consensus sequence, and the clustering of RSTs 2−3, 4−7, 8−11, 14−17, or 20−23 may reflect phylogenetic relationships among these sequences. Therefore, the first RST identifier number reflects the phylogenetic relationships with other RSTs. Furthermore, a comparable discriminative power, but simpler and more economic protocol of RSTing than that of MLST, may indicate the merits of using RSTing [33]. Although the discriminative power of RSTing was less than *spa* typing the antibiotic resistance information from RSTing data may support it being the preferred choice as a frontline tool. Additionally, the higher discriminative power of mPPTing than *spa* typing may also support it being the preferred choice as a second-line tool when combined with RSTing of molecular epidemiology of *S. aureus* infections in animals and humans.

The evolution of *S. aureus* is largely driven by mobile genetic materials such as temperate phages, plasmids, transposons, and mobile pathogenicity islands [29]. Among them, temperate phages have key roles in the transmission and expression of virulence genes of *S. aureus*, and investigations of the prophage profiles of *S. aureus* isolates may be useful in elucidating the molecular evolution and epidemiology of *S. aureus*. Most virulence-related temperate phages possess integrases and are classified into Family *Siphoviridae* [9]. Because integrases are not shared by lytic phages, we chose to investigate terminase large subunit genes common to both lysogenic and lytic phages [3,4,35].

The different frequencies of prophage groups 7 and 9 between bovine isolates and human strains may reflect different kinetics among temperate phages. The absence of prophage groups 5, 6, and 12 and more RSTs without prophages (mPPT0) in bovine isolates than in human strains may reflect a lower probability of phage transduction and less diverse temperate phage pools in bovine isolates. However, the presence of mPPTs possessing at least 5 prophage groups may also reflect frequent phage transduction and diverse temperate phage pools in human *S. aureus* strains.

Among the multi-sampled dairy farms, the frequency of mixed genotypes composed of parent and progeny genotypes of *S. aureus* was more than half (53.8%), and the evolution of bovine isolates by the acquisition of prophages on dairy farms was evident. Additionally, the presence of a single genotype among mastitic cows on a farm should encourage efforts to prevent horizontal transmission. The relatively high frequency of prototypic RST10-2/mPPT0 on different farms (4/26, 15.4%) reflects the method of transmission and introduction onto farms, as well as its role as a founder in the evolution of various mPPTs.

Although LA-MRSA has been reported worldwide, the absence of *mecA* and antibiotics-resistance-related RSTs among bovine isolates in this study reflects a minor potential threat of bovine *S. aureus* transmission to humans [16,22,43]. The presence of virulence genes (*sak* and *cip*) in the mPPT 3 and 7 bovine isolates may be useful for predicting the pathotypes of *S. aureus* isolates.

In this study, we applied RSTing to elucidate the molecular epidemiology of bovine mastitis caused by *S. aureus* and developed mPPTing to evaluate the evolution of RSTs.

According to the results, bovine- and human-specific predominant RSTs of *S. aureus* were identified, and a gradual evolution of *S. aureus* RSTs, via prophage acquisition, was evident on the dairy farms. Thus, a combination of RSTing and mPPTing may be an economic and informative method for understanding the molecular epidemiology of animal and human diseases caused by *S. aureus* infection.

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Conflict of Interest

The authors declare no conflicts of interest.

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Supplementary Table 1. *In silico* typing of *rpoB* sequence types (RSTs) and prophage types (PPTs) of human *Staphylococcus aureus* strains in GenBank

Strain	Accession No.	RST	mPPT	ST	Source
CCARM 3080	KC707788	2-1	2*	5	Korea
CCARM 3A051	KC707799	2-1	2*	5	Korea
CCARM 3A047	KC707798	2-1	2*	NT	Korea
502A	CP007454	2-1	3	1240	–
N315	BA000018	2-1	7-9	5	–
HOU1444-VR	CP012593	2-1	7-9	5	Brazil
ECT-R2	FR714927	2-1	7-9	5	–
10388	HE579059	2-1	7-9	228	–
10497	HE579061	2-1	7-9	228	–
15532	HE579063	2-1	7-9	228	–
16035	HE579065	2-1	7-9	228	–
16125	HE579067	2-1	7-9	228	–
18341	HE579069	2-1	7-9	228	–
18412	HE579071	2-1	7-9	228	–
18583	HE579073	2-1	7-9	228	–
ZJ5499	CP011685	2-1	1-7-9	5	China
UCI62	CP018766	2-1	1-7-9	5	USA
10388	AP009324	2-1	2-7-9	5	–
FDAARGOS_159	CP014064	2-1	2-7-9	UT	USA
SA564	CP010890	2-1	5-7-9	5	Swizerland
FCFHV36	CP011147	2-1	1-2-7-9	105	Brazil
FORC_027	CP012692	2-1	1-2-8-10	5	Korea
UCI 28	CP018768	2-1	7-8-9-10	5	USA
JH1	CP000736	2-1	1-7-8-9-10	105	–
04-02981	CP001844	3-2	7-9	225	–
Mu50	BA000017	3-3	2-7-9	5	–
CCARM 3A052	KC707800	3-4	2-8-10	5	Korea
RIVM6519	CP015173	3-6	7-8-9	5	Nethelands
MI	AP017320	3-7	7-9	5	USA
NCCP14562	CP013955	3-8	1-8-10	5	Korea
VC40	CP003033	4-1	0	8	–
ST20130941	CP012978	4-1	0	15	France
ST20130940	CP012979	4-1	0	15	France
CCARM 3105	KC707790	4-1	1*	247	Korea
ST20130943	CP012974	4-1	3	25	France
ST20130942	CP012976	4-1	3	25	France
LT671859.1	LT671859	4-1	3	8	Germany
JH4899	AP014921	4-1	7-9	8	Japan
V2200	CP007657	4-1	1-3	923	USA
ST20130939	CP012970	4-1	5-10	15	France
ST20130938	CP012972	4-1	5-10	15	France
NCTC8325	CP000253	4-1	1-2-3	8	–
2395 USA500	CP007499	4-1	1-2-3	8	USA
08-02119	CP015645	4-1	1-5-12	582	Germany
USA300_FPR3757	CP000255	4-1	1-7-9	8	–

Supplementary Table 1. Continued

Strain	Accession No.	RST	mPPT	ST	Source
USA300_TCH1516	CP000730	4-1	1-7-9	8	USA
CA15	CP007674	4-1	1-7-9	8	Colombia
HUV05	CP007676	4-1	1-7-9	8	Colombia
UA-S391	CP007690	4-1	1-7-9	8	Belgium
29b MRSA	CP010295	4-1	1-7-9	8	USA
31b MRSA	CP010296	4-1	1-7-9	8	USA
33b	CP010297	4-1	1-7-9	8	USA
26b	CP010298	4-1	1-7-9	8	USA
25b	CP010299	4-1	1-7-9	8	USA
27b MRSA	CP010300	4-1	1-7-9	8	USA
USA300_2014.C01	CP012119	4-1	1-7-9	8	USA
USA300_2014.C02	CP012120	4-1	1-7-9	8	USA
UTSW MRSA 55	CP013231	4-1	1-7-9	8	USA
USA300-SUR12	CP014407	4-1	1-7-9	8	USA
1971.CO1	CP016858	4-1	1-7-9	UT	USA
1969.N	CP016861	4-1	1-7-9	8	USA
1625.CO1	CP016863	4-1	1-7-9	8	USA
2148.CO1	CP017094	4-1	1-7-9	8	USA
C2406	CP019590	4-1	1-7-9	8	Canada
JE2	CP020619	4-1	1-7-9	8	USA
5118.N	CP016855	4-1	2-7-9	UT	USA
JKD6008	CP002120	4-1	7-8-9	239	–
OC8	AP017377	4-1	1-2-7-9	8	Russia
USA300-ISMMS1	CP007176	4-1	1-2-7-9	8	USA
USA300_SUR1	CP009423	4-1	1-2-7-9	8	USA
USA300-SUR1	CP014362	4-1	1-2-7-9	8	USA
USA300-SUR2	CP014365	4-1	1-2-7-9	8	USA
USA300-SUR3	CP014368	4-1	1-2-7-9	8	USA
USA300-SUR6	CP014381	4-1	1-2-7-9	8	USA
USA300-SUR7	CP014384	4-1	1-2-7-9	8	USA
USA300-SUR13	CP014409	4-1	1-2-7-9	8	USA
USA300-SUR14	CP014412	4-1	1-2-7-9	8	USA
USA300-SUR15	CP014415	4-1	1-2-7-9	8	USA
USA300-SUR16	CP014420	4-1	1-2-7-9	8	USA
USA300-SUR17	CP014423	4-1	1-2-7-9	8	USA
USA300-SUR18	CP014426	4-1	1-2-7-9	8	USA
USA300-SUR19	CP014429	4-1	1-2-7-9	8	USA
USA300-SUR20	CP014432	4-1	1-2-7-9	8	USA
USA300-SUR21	CP014435	4-1	1-2-7-9	8	USA
USA300-SUR22	CP014438	4-1	1-2-7-9	8	USA
USA300-SUR23	CP014441	4-1	1-2-7-9	8	USA
USA300-SUR24	CP014444	4-1	1-2-7-9	8	USA
Newman	CP023390	4-1	2-5-7-9	254	USA
M92	CP015447	4-1	3-5-6-12	UT	Canada
M121	CP007670	4-1	1-5-7-9-12	8	–
CCARM 3795	KC707796	4-1	6-7-8-9-10*	239	Korea
V605	CP013959	4-1	6-7-8-9-10	UT	Korea
Tw20	FN433596	4-1	6-7-8-9-10	239	–
V521	CP013957	4-1	5-6-7-8-9-10-12	239	Korea
HC1335	CP012012	5-1	1-2-7-9	239	Brazil

Supplementary Table 1. Continued

Strain	Accession No.	RST	mPPT	ST	Source
Gv88	CP012018	5-1	1-2-7-9	239	Brazil
Gv51	CP012015	5-1	1-5-7-9	239	Brazil
Bmb9393	CP005288	5-1	1-7-8-9	239	Brazil
CCARM 3104	KC707789	5-1	7-8-9-10*	239	Korea
CCARM 3531	KC707792	5-1	7-8-9-10*	239	Korea
Z172	CP006838	5-1	7-8-9-10	239	Taiwan
XN108	CP007447	5-1	5-6-7-9-12	239	China
CCARM 3873	KC707797	5-2	0	72	Korea
CCARM 3724	KC707794	5-2	7-9*	72	Korea
CCARM 3792	KC707795	5-2	7-9*	72	Korea
TMUS2126	AP014652	5-2	7-9	72	Japan
CN1	CP003979	5-2	7-9	72	Korea
2148.N	CP016856	5-2	7-9	NT	USA
FORC_012	CP010998	5-2	2-7-9	72	Korea
CA12	CP007672	5-4	1-7-9	8	USA
DSM 20231	CP011526	5-5	0	8	Japan
M1	HF937103	5-6	1-3	8	Denmark
FDAARGOS_412	CP023500	5-7	7-9	UT	USA
CCARM 3533	KC707793	6-2	6-7-8-9-10*	239	Korea
JH9	CP000703	6-4	1-7-8-9-10	105	–
COL	CP000046	6-5	1	250	–
T0131	CP002643	6-6	7-9	239	–
USA300-SUR4	CP014371	6-7	1-2-7-9	8	USA
USA300-SUR5	CP014376	6-7	1-2-7-9	8	USA
USA300-SUR8	CP014387	6-7	1-2-7-9	8	USA
USA300-SUR9	CP014392	6-7	1-2-7-9	8	USA
USA300-SUR10	CP014397	6-7	1-2-7-9	8	USA
USA300-SUR11	CP014402	6-7	1-2-7-9	8	USA
Gv69	CP009681	6-8	1-5-7-9-12	239	Brazil
HC1340	CP012011	6-8	1-5-7-9-12	239	Brazil
Be62	CP012013	6-10	1-2-7-9	239	Brazil
TMUS2134	AP014653	6-11	7-9	72	Japan
08-02300	CP015646	7-1	7-9	7	Germany
SR434	CP019563	7-1	5-7-9	88	China
ATCC 6538	CP020020	8-1	3	464	Germany
144_S7	CP010943	8-3	7-9	772	South Africa
79_S10	CP010944	8-3	7-9	772	South Africa
DAR4145	CP010526	8-3	2-7-9	772	India
93b_S9	CP010952	9-4	1-2	121	South Africa
GR2	CP010402	10-1	1-3	80	Greece
NCTC13435	LN831036	10-1	1-3	80	UK
RKI4	CP011528	10-1	7-9	27	Germany
11819-97	CP003194	10-1	1-2-3	80	–
FORC_026	CP013132	10-2	0	1	Korea
No.10	AP015012	10-2	1-3	81	Japan
MW2	BA000033	10-2	1-3	1	–
MSSA476	BX571857	10-2	1-3	1	–
FORC_045	CP017115	10-2	1-3	1	Korea
XQ	CP013137	10-6	1-3	121	China
CCARM 3527	KC707791	11-1	7-9*	1	Korea

Supplementary Table 1. Continued

Strain	Accession No.	RST	mPPT	ST	Source
CCARM 3727	KC707803	11-1	7-9*	1	Korea
H-EMRSA-15	CP007659	11-2	1-7-9	22	Belgium
HO 5096 0412	HE681097	11-2	1-7-9	22	GenBank
JKD6159	CP002114	11-3	1-7-9	93	–
71A_S11	CP010940	12-2	3-7-9	22	South Africa
M013	CP003166	14-1	0	59	–
SA957	CP003603	14-1	0	59	Taiwan
SA40	CP003604	14-1	0	59	Taiwan
MS4	CP009828	14-1	0	338	China
HZW450	CP020741	14-1	0	59	China
SA40TW	CP013182	14-1	7-9	UT	China
SA268	CP006630	14-1	5-7-9	59	China
RIVM1607	CP013619	14-2	0	398	Nethelands
S0385	AM990992	14-2	1	398	–
RIVM1295	CP013616	14-2	1	398	Nethelands
GD1677	CP019595	14-2	1	398	China
RIVM3897	CP013621	14-2	1-5	398	Nethelands
293G	CP019591	14-2	7-9	398	Canada
GD705	CP019593	14-2	1-7-9	398	China
6850	CP006706	14-3		50	Germany
ATCC 25923	CP009361	14-4	3	243	USA
G477	CP021905	14-4	3	243	Germany
G478	CP021907	14-4	3	243	Germany
TCH60	CP002110	15-1	1	UT	–
MRSA252	BX571856	15-2	1-7-9	36	
08BA02176	CP003808	15-4	1	398	Canada
71193	CP003045	15-5	7-9	398	
GD5	CP019592	15-7	7-9	398	China
GD1539	CP019594	16-1	1-5-7-9	398	China
JP080	AP017922	20-1	3	UT	Japan
MCRF184	CP014791	21-1	0	45	USA
Tager 104	CP012409	21-2	2-7-9	49	USA
CA-347	CP006044	22-2	1-5-7-9	45	USA
JS395	CP012756	23-1	0	1093	Denmark
SA17_S6	CP010941	25-1	1-7-9	152	South Africa

mPPT, molecular prophage type; ST, sequence type; NT, not tested; UT, untypable. *Result of real molecular prophage typing.