Evolution of a major bovine mastitic genotype (*rpoB* sequence type 10-2) of *Staphylococcus aureus* in cows[§]

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Staphylococcus aureus is the major pathogen leading to bovine mastitis globally while livestock-associated methicillin resistant *S. aureus* (LA-MRSA) has become a potential threat to public health. MRSA from bovine mastitis is not common but a methicillin susceptible *S. aureus* (MSSA) genotype, *rpoB* sequence type (RST)10-2 (RST10-2), is prevalent in Korea. To date, many genomic sequences from *S. aureus* have been elucidated, but the complete genome sequences of RST10-2 MSSA from bovine mastitis has never been reported. In this study, we determined the complete genome sequence of two RST10-2 MSSA that differ from each other in staphylococcal protein A and molecular prophage types [PMB64-1 (t2489/mPPT0) and PMB81-4 (t127/mPPT1-2-3)] and conducted a comparative genomics study. The genomic sequences of PMB64-1 and PMB81-4 were more homologous to the representative human RST10-2 strains (MSSA476, MW2 etc.) compared to other RSTs. Most of them shared five common pseudogenes, along with high amino acid identity of four variable virulence genes that were identified in this study. However, PMB64-1 and PMB81-4 acquired different strain-specific pseudogenes and mobile genetic elements than the human strains. The unique pseudogene profile and high identity of the virulence genes were verified in RST10-2 field strains from bovine mastitis. Thus, bovine mastitic RST10-2 MSSA may have an evolutionary relationship with the human RST10-2 community-associated (CA) MSSA and CA-MRSA strains but may have adapted to cows.

Keywords: *Staphylococcus aureus*, bovine mastitis, comparative genomics, pseudogenes, virulence genes

Introduction

Humans are the original host and act as a reservoir for the

diverse genotypes of *Staphylococcus aureus*. Some human *S. aureus* have adapted to new hosts, like domestic animals and poultry, and have acquired species-specific changes in their genomes (Herron-Olson *et al.*, 2007; Lowder *et al.*, 2009; Guinane *et al.*, 2010; Weinert *et al.*, 2012; Viana *et al.*, 2015). *S. aureus* has evolved by acquiring mobile genetic elements (MGEs), such as lysogenic phages, genomic islands (GIs), staphylococcus cassette chromosomes (SCCs), plasmids, and transposons. Of these, MGEs carried various functional genes encoding virulence factors and antibiotic resistance-related proteins (Fitzgerald *et al.*, 2001). *S. aureus* can survive intracellularly and persist for a long-time in its host, and genes that are unnecessary or cumbersome during infection may be inactivated to form pseudogenes via frame-shift or non-sense mutations. These genes have accumulated in the genome and may help understand the evolutionary track of pathogenic bacteria (Lowy, 2000; Lerat and Ochman, 2005; Herron-Olson *et al.*, 2007). *S. aureus* harbors multiple potent virulence genes for human infection but has lost other virulence genes, possibly during host adaptation (Herron-Olson *et al.*, 2007).

Since 1961, methicillin-resistant *S. aureus* (MRSA) has become one of the most important bacterial threats worldwide and is classified as community-associated (CA), livestock-associated (LA), and hospital-associated MRSA (HA-MRSA) (Jevons, 1961). *S. aureus* causes bovine mastitis and food poisoning, and is an important pathogen in the dairy industry (Sutra and Poutrel, 1994; Le Loir *et al.*, 2003). The genotypes of *S. aureus* from bovine mastitis are variable, but bovine multi-locus sequence type 1 (ST1) MRSA and MSSA strains in Italy share common genetic traits (Battisti *et al.*, 2010; Monaco *et al.*, 2013; Alba *et al.*, 2015). To date, multiple genomic sequences of *S. aureus* from humans, domestic animals, and poultry are available in the GenBank database and the frequency of ST1 was the sixth highest among human strains following ST8, ST239, ST11, ST228, and ST72, in that order (Ko *et al.*, 2018). Recently, we developed a molecular prophage typing (mPPTing) method for *S. aureus* and by combining it with RNA polymerase beta subunit gene (*rpoB*) sequence typing (RSTing), we classified bovine and human *S. aureus* strains (Seong *et al.*, 2013; Ko *et al.*, 2018). The *rpoB* sequence type (RST) 10-2 was the most frequent among *S. aureus* strains from bovine mastitis in Korea, and most of the ST1 strains in GenBank (MSSA476, MW2, FORC 026, FORC 045, etc.) also belonged to RST10-2. Interestingly FORC_026 and FORC_045 were isolated from Korean patients. Therefore, investigation of the genetic linkage between bovine and human RST10-2 strains may be valuable. Furthermore, complete genome sequences of the bovine RST10-2 genotype have never been reported.

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In this study, we determined the complete genome sequence of two bovine RST10-2 strains and conducted a comparative genomics study to understand their evolutionary relationship with human RST10-2 strains and their genetic background of virulence and antibiotic resistance. We also identified the conserved pseudogenes and variable virulence genes among the RST10-2 strains, and verified them in field strains.

Materials and Methods

Bacterial strains

Eight *S. aureus* strains (PMB64-1, PMB81-4, PMB2, PMB46, PMB61, PMB132, PMB232, and PMB235) of RST10-2 and 11 strains of other RSTs [PMB196-1(RST2-1), PMB179-1 (RST4-1), PMB67-1 (RST5-2), PMB8-1 (RST8-1), PMB119-4 (RST10-3), PMB36-1 (RST11-4), PMB146-1 (RST11-5), PMB236-1 (RST11-8), PMB177-1 (RST14-2), PMB65-1 (RST14-3), PMB4-1 (RST22-1)] from bovine mastitis in Korea were reported previously. All strains were cultivated on sheep blood agar (Hangang Co.) (Kim *et al.*, 2017; Ko *et al.*, 2018).

Genome sequencing and assembly

Total DNA from overnight cultures of PMB64-1 and PMB81-4 was extracted using a DNeasy® Blood & Tissue kit (QIAGEN), and PacBio RS II single molecule real time (SMRT) sequencing was performed (Theragen ETS). We also repeated genome sequencing on a HiSeq™ 2000 platform (Illumina), and combined the obtained results. *De novo* as-

sembly was performed using Canu v1.6 (Koren *et al.*, 2017).

Gene prediction and annotation

Prediction of coding sequences (CDS), putative functions, rRNA, tRNA, and tmRNA, was performed using DFAST (<https://dfast.nig.ac.jp/dfc/>; minimum sequence length 200 bp, the CDS prediction tool MetaGeneAnnotator and Prokka; gene annotation was performed using BLAST and HMMER, rRNA prediction was performed using BLAST homology search, and tRNA prediction was performed using Aragom). The pseudogenes of PMB64-1, PMB81-4, and RST10-2 reference strains from human infection [MSSA476 (BX571857), MW2 (BA000033), FORC_026 (CP013132), FORC_045 (CP017175), FDAARGOS_2 (CP026964), FDAARGOS_43 (CP026957), JMUB3031 (AP018923), No. 10 (AP015012), USA-400-0051 (CP019574), FORC59 (CP020354), and JMUB1273 (AP018922)] and from others [CFNSAN007851 (CP017680, bakery environment) and FORC_039 (CP015817, pork meat)] were collected by searching the key words 'frame-shift' and 'stop' in the DFAST result of each strain. We only used pseudogenes that were intact in the reference genome of NCTC-8325 (CP000253) and were common – whether pseudogenes or not – in all compared genomes for analysis. The accession numbers of other reference strains used in this study are represented in Table 4 and Supplementary data Table S5.

Comparison of genome sequences of RST10-2 strains

The GC content and nucleotide homology of PMB64-1 and PMB81-4 genomes were compared with the human reference strains of RST10-2 from Korea (FORC_026 and FORC_

Table 1. Primer sets for PCR and sequencing of pseudogenes and 4 variable virulence genes

Gene	Primer name	Amplicon		
		Sequence (5' to 3')	Location ^a	Size (bp)
WP_000870806.1	Forward 1	CCCTATAGCGGTAAGTACTGACT	111-130	1831
	Forward 2	GCACGTATTGGGTAACGTTAATC	635-657	
	Reverse	AAAACGTCGGCCATGATAGT	1922-1941	
WP_000494956.1	Forward	GGTGGGGAATCAATTGAAAGT	60-80	559
	Reverse	CAGCCTTAATCCAGCAGCTA	599-618	
WP_001790220.1	Forward	TGGTTGTGTATGCATCTCACC	(-29)-(-9)	475
	Reverse	ATGTCGGGGAAATCTTTCAAGT	425-446	
WP_000592610.1	Forward 1	TGTCAAAGATGGTCGTGTCG	33-52	1308
	Forward 2	GGTGGCAATGCACAACACTTT	616-636	
	Reverse	TGATGTCTACGCGCTAATTCC	1320-1340	
WP_001834510.1	Forward	CCGTTTCAGGTATCATCTTCATTTTATTC	(-78)-(-50)	433
	Reverse	GCAATACAAAAAGCGAGTTAATG	(+29)-(+52)	
<i>set16 (ssl1)</i> ^b	Forward	GAAATTTAAAGCGATAGCAAAAGC	3-26	657
	Reverse	TTTTGTATTGTCTGCCATCG	639-659	
<i>set19 (ssl4)</i>	Forward	GCTAAAACAAGTTTAGCACTAGG	19-41	896
	Reverse	GTGCCATCTATGACATCTGC	895-914	
<i>set26 (ssl11)</i>	Forward	GCTAAAGCAAGTTTAGCACTAG	19-40	637
	Reverse	CAATCGTATCACCCATGCGAT	635-655	
<i>coa</i> ^c	Forward 1	CAAATAATTTGCTAGGCGC	10-29	1893
	Forward 2	GAGTTACGAATCAACGTACTGAA	689-711	
	Reverse	TTATTTTGTACTCTAGGCC	1882-1902	

^a The location of primer is numbered from start codon. The upstream of start codon and downstream of stop codon were represented as – or +.

^b *set*, Staphylococcal exotoxin.

^c *coa*, Coagulase

Table 2. General genomic features of PMB64-1 and PMB81-4

	PMB64-1	PMB81-4
Accession no.	CP034486	CP034441
ST ^a	1	1
<i>sta</i> ^b	t2459	t127
RST/mPPT ^c	10-2/0	10-2/1-2-3
Length (bp)	2,775,972	2,876,590
GC content (%)	32.90	32.86
No. of CDS ^d	2,609	2,756
Depth	696	720
tRNA	61	60
rRNA	19	19
5S	7	7
16S	6	6
23S	6	6
tmRNA ^e	1	1

^a ST, Sequence type determined by multi-locus sequence typing.

^b *sta*, Staphylococcal protein A type.

^c RST/mPPT, *rpoB* sequence type/molecular prophage type.

^d CDS, Coding sequence.

^e tmRNA, Transfer-messenger RNA.

045), and other countries (MSSA476, MW2, and No. 10) in GenBank, using BRIG 0.95 (<http://sourceforge.net/projects/brig/>). Genome sequence similarity was compared by BLASTN

search and the most identical genome sequence was selected based on both the max score and the total score of the search results.

In silico analysis of MGEs

The prophages present in the genome sequences were analyzed using PHASTER (Arndt *et al.*, 2016). Whole nucleotide sequences of known GIs in *S. aureus* were compared with the genome sequences of PMB64-1 and PMB81-4 by BLASTN search (Table 4), and SCCmec was searched using SCCmecFinder 1.2 (Kaya *et al.*, 2018).

Prediction of virulence and antibiotic resistance genes of PMB64-1 and PMB81-4

The presence of virulence genes was predicted by comparing the reported virulence genes of VFDB (<http://www.mgc.ac.cn/cgi-bin/VFs/genus.cgi?Genus=Staphylococcus>) with the genome sequences of PMB64-1 and PMB81-4 by a BLASTN search. Antibiotic resistance genes were identified using CARD 3.0.0 (the comprehensive antibiotic resistance database) and RGI (Resistance Gene Identifier) 4.2.2 software for prediction of the resistome based on homology and SNP models (<https://card.mcmaster.ca/>) (McArthur *et al.*, 2013).

Fig. 1. Comparison of PMB64-1 and PMB81-4 genome sequences with other human RST10-2 reference strains, FORC_026, FORC_045, MSSA476, MW2, and No. 10. BRIG 0.95 (<http://sourceforge.net/projects/brig/>) was used for genome comparison. The different mobile genetic elements unique to PMB64-1 and PMB81-4 are represented around the circular map of genomes.

Table 3. Comparison of predicted prophages of RST10-2 isolates and strains

Most common phage (Accession no.)	PMB81-4	FORC_045 ^b	MSSA476	MW2	No.10 ^c	FDAARGOS_43 ^d	FORC_039
φTEM123 (NC_017968)	140, 50.2 kb, 33.62%, 38/61, 1089267-1139552 ^a	-	-	-	-	-	-
φYMC/09/04/R1988 (NC_022758)	150, 61.4 kb, 32.86%, 26/73, 1554189-1615613	+	-	-	-	-	-
φJS01 (NC_021773)	130, 55.5 kb, 32.09%, 34/58, 2073608-2129151	+	+	+	+	+	-
φIPLA35 (NC_011612)	-	-	+	-	-	-	-
φ7401PVL (NC_020199)	-	-	-	+	-	-	-
φ2958PVL (NC_011344)	-	-	-	-	-	+	-
φNM3 (NC_008617)	-	-	-	-	-	-	+
φStauST398 (NC_021326)	-	-	-	-	-	-	+

^a Score, region length, GC content, no. of hit/total protein, location^b FDAARGOS_2, JMUB3031^c CFSAN007851^d USA400-0051

PCR and sequencing for characterization of pseudogenes and virulence genes of RST10-2 field strains

The primer sets used to amplify the five common pseudogenes and four variable virulence genes were newly designed for this study and are listed in Table 1. When some strains did not show amplicons with the forward 1 primer, the forward 2 primer was used for PCR. To extract the total DNA of *S. aureus* strains, bacteria were grown overnight in LB broth at 37°C, and the G-spinTM for Bacteria Genomic DNA Extraction Kit (iNtRON Biotechnology) was used according to the manufacturer's instructions. The PCR mixture was comprised of 5 µl of 10X reaction buffer, dNTPs (2 mM, 5.0 µl), MG Max Taq DNA polymerase (5 U/µl; MGmed; 0.5 µl), forward and reverse primers (1. 25 µM, 1.25 µl of each), distilled water (36.5 µl), and template DNA (50 ng/µl, 1 µl). The PCR mixture was incubated at 95°C for 5 min followed by 35 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 1–2 min; and a final extension step at 72°C for 5 min. The PCR amplicons were purified using the MG PCR Purification kit (MGmed) and sequenced using PCR primers on an ABI3711 automatic sequencer (Macrogen Co.). The

determined partial sequences were compared with the corresponding amino acid sequences using translated BLAST (blastx).

Antimicrobial susceptibility testing

Antimicrobial susceptibility of PMB 64-1 and PMB 81-4 was performed according to the CLSI guideline (M100S, 26th edition) for 21 antimicrobial agents (ampicillin, amoxicillin/clavulanic acid, chloramphenicol, ciprofloxacin, doxycycline, gentamicin, kanamycin, norfloxacin, penicillin, sulfamethoxazole/trimethoprim, tetracycline, cefazolin, ceftiofur, florfenicol, colistin, enrofloxacin, ofloxacin, levofloxacin, linezolid, erythromycin, and fusidic acid) by disc diffusion and gentamicin by microbroth dilution method, respectively (Seong *et al.*, 2013).

Statistical analysis

The frequencies of pseudogenes of *S. aureus* strains were compared via chi-squared and Fisher's exact tests (95% CI) using SPSS for Windows, ver. 22.0 (SPSS).

Table 4. Comparison of genomic islands of RST10-2 isolates and strains

Class of islands	Name of island	Strain/accession no.	PMB64-1	PMB81-4 ^a	MW2 ^b	No.10	FORC_039	FORC59 ^c
vSaα	-	MSSA476	+ (418396-450447) ^d	+ (419621-451682)	+	+	+	+
vSaβ	-	USA300 TCH1516/CP000730	+ (1873381-1910662)	+ (1924865-1959467)	+	+	-	-
vSaγ	-	"	+ (1136725-1156524)	+ (1146986-1166953)	+	+	+	+
vSa1	SaPI5	"	+ ^e (881787-895515)	+ (849355-863292)	+	+	+	+
	SaPI-3	COL/CP000046	-	-	-	-	-	-
vSa2	SaPIbov1	RF122/AJ938182	-	-	-	-	-	-
vSa3	SaPI _{Im} 4	Mu50/BA000017	-	-	-	-	-	-
vSa4	SaPI _{Im} 1	"	-	-	-	-	-	-
	SaPI6Δ	USA300 TCH1516	+ (2049325-2052466)	+ (2135002- 2138143)	+	-	-	-
vSa5	SaPI4	MRSA252/BX571856	-	-	-	-	-	-
SCC _{fur}	-	MSSA476	-	+ (56984-34149)	-	-	-	-
SCC _{mec}	-	MW2	-	-	+ ^f	-	+ ^f	-

^a PMB81-4 = MSSA476 = FORC_026 = FORC_045 = FDAARGOS_2^b MW2 = FDAARGOS_43 = CFSAN007851 = USA400-0051 = JMUB3031 = FORC_039^c FORC59 = JMUB1273^d Location^e 97% coverage/99% identity (others are 99–100% coverage/99–100% identity)^f SCC_{mec} type IVa(2B)

Results

General genomic features of the bovine ST1/RST10-2 MSSA strains, PMB64-1, and PMB81-4

The GC content of PMB64-1 and PMB81-4 was similar but the genome size of PMB64 (2,775,972 bp) was smaller than that of PMB81-4 (2,876,590 bp) (Table 2). The CDS number of PMB64-1 (2,609) was less than that of PMB81-4 (2,756) but the number of tRNAs, rRNAs (5S, 16S, and 23S), and tmRNAs in PMB64-1 was similar to that in PMB81-4 (Table 2). The genome sequences of PMB64-1 and PMB81-4 were more similar to the RST10-2 reference strain, MSSA476 (max scores 5.321e+05 and 9.742e+05, and total scores 5.721e+06 and 5.903e+06, respectively). Figure 1 shows the comparison of the genome sequences of PMB64-1 and PMB81-4 with the RST10-2 reference strains, FORC_026, FORC_045, MSSA476, MW2, and No. 10. The Korean MSSA strains, FORC_026 and FORC_045, were previously deposited in the GenBank database by the FORC (Food-borne pathogen Omics Research Center) in Korea.

Molecular characterization of MGEs of PMB64-1 and PMB81-4

In a previous study, PMB64-1 and PMB81-4 showed different prophage types, mPPT0 and mPPT1-2-3, respectively; we verified the difference in the prophage profile (Ko *et al.*, 2018). PMB64-1 was predicted to possess no prophage but PMB81-4 did show three intact prophages (ϕ TEM123-like, ϕ YMC/09/04/R1988-like, and ϕ JS01-like prophages). Although the three prophages of PMB81-4 showed the highest similarity, they were unique enough to differ from ϕ TEM123, ϕ YMC/09/04/R1988, and ϕ JS01 due to a low coverage percentage and the number of hit proteins out of the total proteins. The genetic information of the prophages is summarized in Table 3. Among the three prophages, the ϕ JS01-like prophage was inserted into the β -hemolysin gene (*hly*) resulting in loss of the β -hemolysis trait in PMB81-4. The

ϕ JS01-like prophage was shared by PMB81-4, FORC-045, MSSA476, MW2, No. 10, CFSAN007851, FDAARGOS_2, FDAARGOS_43, USA400-0051, and JMUB3031, whereas the ϕ YMC/09/04/R1988-like prophage was shared by PMB81-4, FORC_045, FDAARGOS_2, and JMUB3031. We confirmed the presence of the ϕ JS01-like prophage in the RST10-2 reference strains by querying the nucleotide sequences (2073608-2129151) of PMB81-4 using BLASTN. According to the result, all the RST10-2 reference strains possessing a ϕ JS01-like prophage completely matched with the queried sequence. Therefore, it was thought that the same ϕ JS01-like prophage might be shared by most RST10-2 strains.

To date, various GIs and SCCs have been identified in the genomes of *S. aureus* and we have identified these in the PMB64-1 and PMB81-4 genomes (Table 4). The GI contents of PMB64-1 and PMB81-4 were similar to that of the other RST10-2 strains and they possessed *vSa*, *vSa* β , *vSa* γ , *vSa*1 (*Sa*P15), and *vSa*4 (*Sa*P16 Δ). PMB64-1 and PMB81-4 did not possess *SCCmec* (genotype IVa) that is present in MW2, FDAARGOS_43, CFSAN007851, USA400-0051, JMUB3031, and FORC_039, while PMB81-4, FORC_026, FORC_045, MSSA476, and FDAARGOS_2 contains *SCCfur*, which is a fusidic acid resistance gene. No. 10, FORC59, and JMUB1273 did not possess either *SCCfur* or *SCCmec* but they are different from other RST10-2 strains in *vSa* β and *Sa*P16 Δ .

We identified insertional sequences (ISs) of PMB64-1 and PMB81-4 genomes and found a high number (35 intact and 2 nonfunctional by mutations) of IS256 in PMB64-1 whereas these were absent in PMB81-4. None of the RST10-2 strains except FORC_045 (13 intact and 1 nonfunctional IS256) possessed IS256 in their genomes. Among the IS256 loci, some were expected to form composite transposons that may carry several genes encoding virulence factors, antibiotic resistance, and hypothetical proteins (Supplementary data Table S1). Particularly, *Tn4001*, flanked by IS256 and containing an aminoglycosides resistance gene, *aac*(6')-Ie-aph(2'')-Ia and N-acetyl transferase was identified.

Table 5. Comparison of virulence gene frequency of RST10-2 isolates and strains

Strain	Category of virulence factors ^a						
	Adherence (19) ^a	Exo-enzyme (15)	Immune evasion (16)	Secretion system (8)	Toxin (67)	No. of intact gene (125)	No. of pseudogene (%)
PMB64-1	17/1 ^b	12/1	14/2	8/0	20/1	71	5 (6.6)
PMB81-4	18/1	14/1	16/0	8/0	23/1	79	3 (3.7)
FORC_026	17/2	11/1	16/0	8/0	21/0	73	3 (3.9)
FORC_045	17/2	15/0	11/1	8/0	23/1	74	4 (5.1)
MSSA476	16/1	13/0	16/0	8/0	21/1	74	2 (2.6)
MW2	18/1	13/0	16/0	8/0	27/1	82	2 (2.4)
No.10	18/1	15/0	16/0	8/0	25/2	82	3 (3.5)
CFSAN007851	19/0	15/0	16/0	8/0	25/2	83	2 (2.4)
FDAARGOS_2	19/0	15/0	16/0	8/0	24/1	82	1 (1.2)
FDAARGOS_43	19/0	15/0	16/0	8/0	28//2	86	2 (2.3)
USA 400-0051	18/1	13/2	16/0	8/0	28/2	83	5 (5.7)
JMUB 3031	19/0	15/0	16/0	8/0	23/1	81	1 (1.2)
FORC_039	19/0	15/0	16/0	5/1	19/1	74	2 (2.6)
FORC59	19/0	15/0	16/0	5/1	20/0	75	1 (1.3)
JMUB 1273	19/0	15/0	16/0	6/0	18/1	74	1 (1.3)

^a No. of compared genes from VFDB (<http://www.mgc.ac.cn/cgi-bin/VFs/genus.cgi?Genus=Staphylococcus>)

^b No. of intact gene/no. of pseudogene

Table 6. Amino acid coverage and identity percent of 4 highly variable virulence genes of RST10-2 in *S. aureus* isolates and strains

Virulence gene	Major RST10-2 ^a	Minor RST10-2		Other RSTs ^c
		JMUB3031	FORC_039 ^b	
<i>set16</i> (<i>ssl1</i>)	100/100 ^d	100/100	100/100	100/68-93
<i>set19</i> (<i>ssl4</i>)	100/99-100	65/88 ^e	100/99	67-100/67-93
<i>set26</i> (<i>ssl11</i>)	100/99-100	100/100	100/99	99-100/61-94
<i>coa</i>	100/99-100	100/99	100/90	99-100/67-91

^a Major RST10-2: PMB64-1, PMB81-4, FORC_026, FORC_045, MSSA476, MW2, No.10, CFSAN007851, FDAARGOS_2, FDAARGOS_43, USA400-0051.

^b FORC_039 = FORC59 = JMUB1273

^c Other RSTs such as in Supplementary data Table S5 except *set16* of PMB65-1 (RST14-3, 100%), PMB146-1 (RST11-5, 100%), PMB208-1 (RST11-6, 99%), and PMB236-1 (RST11-8, 100%); except *set26* of PMB146-1 (100%), PMB208-1 (97%), and PMB236-1 (100%); except *coa* of PMB236-1 (99%)

^d % amino acid coverage/% amino acid identity

^e Pseudogene

Comparison of virulence genes of PMB64-1 and PMB81-4 with the reference strains of RST10-2

The 125 virulence genes categorized into adherence (19), exoenzyme (15), host immune evasion (16), secretion system (8), and toxin (67) (VFDB: <http://www.mgc.ac.cn/cgi-bin/VFs/>) were compared between the genome sequences of PMB64-1 and PMB81-4. The presence or absence of virulence genes was compared with all the RST10-2 reference strains (Table 5 and Supplementary data Table S2). PMB64-1 and PMB81-4 possessed 71 and 79 intact virulence genes, and 5 (*ebh*, *splD*, *capD*, *capO*, and *hla*) and 3 (*eap*, *geh*, and *hlyB*) pseudogenes, respectively, and they showed different numbers and pseudogene frequency (Table 5). Although the difference was not significant, the frequency (6.6%) of pseudogene of PMB64-1 was slightly higher than other strains such

as FDAARGOS_2 and JMUB3031 (1.2%). PMB81-4 possessed additional virulence genes related to adherence (*fmbB*), colonization (*sak*), thermonuclease (*nuc*), and enterotoxins (*sea*, *selk*, and *selq*) compared to PMB64-1 (Table 5 and Supplementary data Table S2). However, the human CA-MRSA strain, FDAARGOS_43 possessed the highest number of virulence genes (86) among the compared RST10-2 strains.

Identification of highly variable virulence genes conserved among the RST10-2 genotypes

Of the conserved virulence genes among RST10-2 strains, highly variable genes between RSTs were identified (Table 6). The four highly variable genes, staphylococcal exotoxins 16, 19, 26, and coagulase genes (*set16*, *set19*, *set26*, and *coa*) were conserved among RST10-2 strains (100% amino acid

Table 7. Comparison of common and total pseudogenes of RST10-2 isolates and strains

Strain	WP_000870806	WP_000494956	WP_001790220	WP_000592610	WP_001834510	Total no. of Pseudogene (%) ^a
	Bacteriocin-associated integral membrane protein	ImmA/IrrE family metallo-endopeptidase	Transposase for IS232	Imidazole glycerol phosphate synthase	Fibronectin binding protein	
PMB64-1	P ^b	P	P	P	P	48 (32.0)*
PMB81-4	P	P	P	P	P	35 (23.3)*
PMB2	P	P	P	P	P	nt ^d
PMB61	P	P	P	P	P	nt
PMB132	P	P	P	P	P	nt
PMB232	P	P	P	P	P	nt
PMB235	P	P	P	P	P	nt
FORC 026	P	P	P	P	P	14 (9.3)
FORC 045	P	P	P	P	P	22 (14.7)
MSSA 476	P	P	P	P	P	14 (9.3)
MW2	P	P	P	P	P	8 (5.3)
No. 10	P	P	P	P	P	7 (4.7)
CFSAN007851	P	P	P	P	P	13 (8.7)
FDAARGOS_2	P	P	P	P	P	14 (9.3)
FDAARGOS_43	P	P	P	P	P	9 (6.0)
JMUB3031	P	P	P	P	P	21 (14.0)
PMB46	I ^c	P	P	P	P	nt
USA400-0051	P	P	I	I	P	26 (17.3)
FORC_039	I	I	I	P	I	9 (6.0)
FORC59	I	I	I	P	I	15 (10.0)
JMUB1273	I	I	I	P	I	10 (6.7)

^a % of pseudogene [no. of pseudogene/total no. of compared gene (150) × 100]

^b P: pseudogene

^c I: intact gene

^d nt: not tested

* Significant frequency difference from No.10.

Table 8. Comparison of antibiotic resistance genes of PMB64-1 and PMB81-4 with other human RST10-2 strains

Gene	Resistant antibiotics	Resistance mechanism	PMB 64-1	PMB 81-4	FORC 026	FORC 045	MSSA 476 ^a	MW 2 ^b	No. 10 ^c	JMUB 3031 ^d
<i>aac(6')-Ie-aph(2'')-Ia</i>	Aminoglycosides	Inactivation	+	-	-	+	-	-	-	-
<i>arlR</i>	Fluoroquinolones, acridine dye	Efflux	+	+	+	+	+	+	+	+
<i>arlS</i>	Fluoroquinolones, acridine dye	Efflux	+	+	+	+	+	+	+	+
<i>ermA</i>	Macrolides, lincosamides, streptogramin	Target alteration (23S rRNA methylation)	-	+	+	-	-	-	-	-
<i>fusC</i>	Fusidic acid	Inactivation	-	+	+	+	+	-	-	-
<i>gyrA</i>	Fluoroquinolone	S84L(SNP)	-	-	-	-	-	-	-	+
<i>mecA</i>	Monobactam, penam, cephalosporin, cephamycin, carbapenem	Methicillin resistant PBP2	-	-	-	-	-	+	-	+/-
<i>mepA</i>	Tetracyclines, glycylicycline	Efflux	+	+	+	+	+	+	+	+
<i>mepR</i>	Tetracyclines, glycylicycline	Efflux	+	+	+	+	+	+	+	+
<i>mgrA</i>	Tetracyclines, fluoroquinolones, penam, antibiotic peptides, cephalosporin, acridine dye	Efflux	+	+	+	+	+	+	+	+
<i>parC</i>	Fluoroquinolone	S80F and/or E84G(SNP)	-	-	-	+	-	-	-	+
<i>tet(38)</i>	Tetracyclines	Efflux	+	+	+	+	+	+	+	+

^a MSSA476 = FDAARGOS_2^b MW2 = FDAARGOS_43 = USA400-0051^c No.10 = CFSAN007851 = JMUB1273^d JMUB3031 = FORC_039 = FORC59 (*mecA*-)

coverage and 99–100% amino acid identity) but were variable among other RSTs (67–100% amino acid coverage and 61–94 amino acid identity). We verified the conservation and variability of the four virulence genes in Korean field strains. According to the obtained results, the four variable virulence genes of RST10-2 strains, PMB2, PMB61, PMB132, PMB232, and PMB235, showed high amino acid identity (99–100%), but other field strains of different RSTs [PMB4-1 (RST22-1), PMB8-1 (RST8-1), PMB36-1 (RST11-4), PMB-67-1 (RST5-2), PMB119-4], PMB177-1 (RST14-2), and PMB-196-1 (RST2-1)] showed relatively low identity to those of

PMB64-1, PMB81-4 and RST10-2 reference strains, or generated no amplicon after PCR except some virulence genes in some field strains (Table 6)

Identification of conserved pseudogenes among RST10-2 genotype

The pseudogenes of PMB64-1, PMB81-4, and the reference strains of RST10-2 were identified using the DFAST program and were compared to identify the common and strain-specific pseudogenes. The five common pseudogenes shared

Fig. 2. A schematic dendrogram for summary of genetic relatedness of RST 10-2 strains. Firstly, the RST10-2 strains were divided into 3 clusters by the numbers of common pseudogenes, and secondly the presence of φ JS01 and absence of prophages. The discriminative genetic markers and characteristics of each RST10-2 strain were also represented in the right columns for further differentiation.

by the most RST10-2 strains are listed in Table 7, and other partially common and strain-specific pseudogenes are summarized in Supplementary data Table S4. A major pseudogene group sharing five common pseudogenes was identified. The putative functions of pseudogenes are described in Table 7. The total number of pseudogenes was different among the RST10-2 strains, and PMB64-1 showed the highest number and frequency (48 and 32.0%) followed by PMB81-4 (35 and 23.3%) and FORC_045 (22 and 14.7%) among RST10-2 strains sharing the five common pseudogenes. However, No. 10 (7 and 4.7%) showed the least number and frequency of pseudogenes followed by MW2 (8 and 5.3%) and FDAARGOS_43 (9 and 6.0%) (Table 7). The pseudogene frequency of PMB64-1 and PMB81-4 was significantly different from that of No. 10 ($P < 0.05$). The five common pseudogenes were conserved in the RST10-2 field strains, PMB2, PMB61, PMB132, PMB232, and PMB235, but PMB2 contained an intact WP_000870806. Therefore, the 5 pseudogenes were conserved in 15 out of 20 RST10-2 strains (15/20, 75%). Additionally, other RSTs from bovine mastitis showed different [PMB196-1 (RST2-1), PMB67-1 (RST5-2), PMB8-1 (RST8-1), PMB177-1 (RST14-2), and PMB65-1 (RST14-3)] or similar pseudogene profiles [PMB179-1 (RST4-1)] as those in human corresponding RSTs (Supplementary data Table S5). However, some of them were incomparable due to the absence of corresponding human strains [PMB119-4 (RST10-3), PMB6-1 (RST11-4), PMB146-1 (RST11-5), PMB208-1 (RST11-6), PMB236-1 (RST11-8), and PMB4-1 (RST22-1)].

In silico prediction of antibiotic resistance genes and antibiotic resistance of PMB64-1 and PMB81-4

The antibiotic resistance genes [*tet*(38), *mepA*, *mepR*, *mgrA*, *arlS*, and *arlR*] encoding pumps to efflux tetracyclines, fluoroquinolones, and cephalosporins were identified in both PMB64-1 and PMB81-4, but *aac*(6')-Ie-aph(2'')-Ia and *fusC*, and *ermA* genes conferring resistance to multiple aminoglycosides and fusidic acid, and macrolides were identified in only PMB64 and PMB81-4, respectively (Table 8). PMB64-1 and FORC_045 showed the same antibiotic resistance gene profile except for *fusC*, whereas PMB81-4 and FORC_026 showed the same profile. MSSA476 showed the same profile as that of PMB81-4 and FORC_026 except for *ermA*. The antibiotic resistance tests showed that PMB 64-1 and PMB 81-4 were resistant to colistin. In addition, PMB 64-1 was resistant to gentamicin (MIC 64 µg/ml) and kanamycin, while PMB 81-4 was resistant to fusidic acid and erythromycin. However, PMB64-1 and PMB81-4 showed susceptibility to tetracycline/oxytetracycline and all tested quinolones.

Discussion

Recently, a high frequency of RST10-2 among bovine mastitis *S. aureus* strains in Korea was reported, and they were determined to be ST1 by MLST (Ko *et al.*, 2018). Considering that the frequency of RST10-2 among human strains of which whole genome sequences were deposited in GenBank was not high, a relatively high rate of Korean strains is present among RST10-2 (4/12, 33.3%), which is noteworthy (Table 6). In addition, the relatively high nucleotide iden-

tity of PMB64-1 and PMB81-4 genomes with RST10-2 human strains including MSSA476 may support an evolutionary relationship between them (Herron-Olson *et al.*, 2007; Sakwinska *et al.*, 2011).

To date, various virulence genes have been identified in *S. aureus* and these are horizontally transmitted by MGEs. Prophages transfer virulence genes of their own and others by providing phage coats (Novick *et al.*, 2010; Moon *et al.*, 2015, 2016). As expected by molecular prophage typing, PMB81-4 contained three putative prophages, whereas PMB64-1 and FORC_026 did not contain any. Among them the ϕ JS01-like prophage was shared by FORC-045, MSSA476, MW2, No. 10, CFSAN007851, FDAARGOS_2, FDAARGOS_43, JMUB-3031, and USA400-0051, whereas the ϕ YMC/09/04/R1988-like prophage was only shared by PMB81-4 and FORC_045. Interestingly, ϕ TEM123 and ϕ YMC/09/04/R1988 were identified as temperate and lytic phages, respectively, of wild type *S. aureus* in Korea, and the presence of ϕ TEM123-like and ϕ YMC/09/04/R1988-like prophages in PMB81-4 and FORC_045 may reflect common exposure of bovine and human *S. aureus* to similar phages in Korea (Jeon *et al.*, 2014). MSSA-476 was isolated from a boy suffering osteomyelitis and regarded as community-acquired MSSA (CA-MSSA) (Holden *et al.*, 2004). ϕ JS01 was identified in the raw milk of a cow in China and ϕ SauS-IPLA35 was isolated from a dairy environment (Garcia *et al.*, 2009; Jia *et al.*, 2013). Therefore, the presence of ϕ JS01-like and ϕ SauS-IPLA35-like prophages in the MSSA476 genome may also support an evolutionary human-bovine connection of RST10-2 *S. aureus*. However, the human CA-MRSA strains, MW2 and FDAARGOS_43 possessed different prophages that were more common with ϕ 7401PVL and ϕ 2958PVL containing Pantone-Valentine leukocidin genes (Ma *et al.*, 2008; Mariem *et al.*, 2013).

Out of the tested GIs, vSaa, vSay, and SaPI5 were the most common but vSa β , SaPI6 Δ , and SCCs were randomly possessed by the RST10-2 strains. Therefore, different classes of GIs may have been acquired at different time points during *S. aureus* evolution. Interestingly the high copy numbers of IS256 and the presence of Tn4001 in PMB64-1 and FORC_045 was in contrast with its absence in other RST10-2 strains. However, the fact some *S. aureus* strains in other countries possess multiple IS256 (data not shown) may reflect the common evolutionary features of *S. aureus* worldwide.

Various GIs were involved in the virulence and antibiotic resistance transmission. Exotoxins (*set*) and lipoprotein like clusters (*lpl*), serine proteases (*splA-F*) and leukocidins (*lukD* and *lukE*), and fibrinogen binding protein (*efb*) genes were located on vSaa, vSa β , and vSay, respectively (Baba *et al.*, 2002, 2008; Gill *et al.*, 2005; Novick *et al.*, 2010; Hiramatsu *et al.*, 2013; Nguyen *et al.*, 2015). vSaa and vSa β transmitted horizontally and evolved independently of the *S. aureus* genome. They were classified into different genotypes based on the *hdsS* gene playing a role in site-specific methylation of the restriction-modification system (Baba *et al.*, 2008). Virulence proteins can be targets of host immunity and hypervariable virulence proteins can be good candidates for vaccine development. We compared the amino acid identity of all virulence genes in different RSTs and found four highly variable virulence genes. Interestingly, three exotoxins on vSaa were selected as hypervariable but unique to most RST-

10-2 strains. Therefore, the relatedness of hypervariability of gene contents of vSaa and host immune response may be one of valuable future studies for vaccine development.

Although the phenotypic effects of the five conserved pseudogenes are not demonstrated, their presence in most of the RST10-2 strains may reflect an evolutionary relationship among them. In addition, the similar or different 5-pseudogene-profiles between bovine strains and human strains in other RSTs may support the potential usefulness of these five pseudogenes as molecular markers. Furthermore, the presence of different but unique 5-pseudogene-profiles among RST10-2 strains (FORC_039, FORC59, and JMUB1273) may reflect the presence of different evolutionary pathways that can be speculated based on the prevalence of different ST1 genotypes in pigs (Battisti *et al.*, 2010; Monaco *et al.*, 2013). The number of pseudogenes may reflect the evolutionary pathway of each *S. aureus* strain. The least number of pseudogenes in human CA-MRSA strains in contrast to PMB64-1 and PMB81-4 may support the increase in pseudogenes during the bovine adaptation of human *S. aureus* (Herron-Olson *et al.*, 2007). However, the different numbers and patterns of pseudogenes among human strains may be useful as molecular markers to unravel the molecular epidemiology of *S. aureus* infections.

The methicillin resistance transferred by SCCmec was not observed in PMB64-1 and PMB81-4. SCCfur and SCCmec are integrated at the integration site sequence of *orfX* in the *S. aureus* genome and SCCfur-bearing PMB81-4 may have a low opportunity to acquire SCCmec (IWG-SCC, 2009). However, PMB64-1 and No. 10 have empty integration sites that can be occupied by SCCmec, and checking this point needs to be considered during molecular epidemiological studies. The acquisition of Tn4001 carrying the *aac(6')-Ia* gene that confers resistance to a high concentration of aminoglycosides, and high copy numbers of IS256 in PMB64-1 and FORC_045 may imply an ongoing evolution in cows and the presence of a common niche to exchange MGEs between human and bovine *S. aureus* (Lyon *et al.*, 1984).

Thus, the absence of prophages and SCCs but the presence of more pseudogenes and IS256 in PMB64-1 may provide hints to delineate the evolutionary steps of the human ancestor of RST10-2 during bovine adaptation. The major genotype of RST10-2 strains was characterized by four hyper-variable virulence genes and five pseudogenes in common, and the distribution of the RST10-2 major genotype among cows and humans may support their close evolutionary relationship (Fig. 2). Although the host shift from humans to cows was concurrent with the loss of function in human colonization (Sakwinska *et al.*, 2011), molecular inspection of human infection and SCCmec acquisition by RST10-2 LA-MSSA needs to be encouraged.

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