表　　題　　オキサシリン感性 *mecA* 陽性黄色ブドウ球菌におけるβラクタム薬高度耐性化機構の解明
(Mechanisms of acquisition of β-lactam resistance in oxacillin-susceptible *mecA*-positive *Staphylococcus aureus*)

論 文 の 区 分　　博士課程

著　　者　　名　　ブーンシリ タニト
Boonsiri Tanit

担当指導教員氏名　　教授　　崔　龍洙

所　　属　　自治医科大学大学院医学研究科
人間生物学　　専攻
生体防御医学　　専攻分野
微生物・免疫学　専攻科

令和 2 年 1 月 10 日申請の学位論文

CONTENTS

1. Abstract

Staphylococcus aureus strains that are susceptible to the β-lactam antibiotic oxacillin despite carrying *mecA* (oxacillin-susceptible methicillin-resistant *S. aureus* [OS-MRSA]) cause serious clinical problems globally because of their ability to acquire β-lactam resistance. Understanding the genetic mechanism(s) responsible for the acquisition of β-lactam resistance in OS-MRSA is therefore crucial for its clinical management. For this purpose, whole-genome sequencing-based analysis was performed using clinical OS-MRSA isolates and their oxacillin-resistant mutants. Genomic analysis revealed extensive genomic diversity among 43 OS-MRSA strains (11 MLST types and 4 SCC*mec* types) and identified 118 variants of 141 mutations in 46 genes and 8 intergenic regions among the 100 oxacillin-resistant mutants generated from 26 OS-MRSA strains. Among them, mutations were most frequently found in genes related to RNA polymerase (*rpoBC*), purine biosynthesis (*guaA*, *prs*, *hprT*, *rsh*), glycolysis (*pykA*, *fbaA*, *fruB*), protein quality control (*clpXP*, *ftsH*), and tRNA synthase (*lysS*, *gltX*), whereas no mutations existed in the *mec* and *bla* operons. The whole-genome transcriptional profile of the resistant mutants demonstrated that downregulation of genes associated with purine biosynthesis, protein quality control, and tRNA synthesis, mimicking the classic stringent response, decreased intercellular

GTP levels and targeted metabolic pathways toward the induction of *mecA* expression by upregulating *blaR1* and altering the expression of genes involved in autolysis and peptidoglycan synthesis. Moreover, the resistance mutations exhibited a relatively low fitness cost, explaining the easy selection of β-lactam–resistant OS-MRSA mutants during the course of antimicrobial treatment.

Keywords: oxacillin-susceptible *mecA*-positive *Staphylococcus aureus*; MRSA; OS-MRSA; *Staphylococcus aureus*; β-lactam resistance; transcriptome; stringent response; (p)ppGpp; drug resistant mutant; whole genome sequence

Abbreviations

ATP: Adenosine triphosphate

CLSI: Clinical and Laboratory Standards Institute

DNA: deoxyribonucleic acid

dNTP: Deoxyribonucleotide triphosphate

GDP: Guanosine diphosphate

GTP: Guanosine triphosphate

HP: Hypothetical protein

IR: Intergenic region

LB: Luria-Bertani

MHA: Mueller–Hinton agar

MHB: Mueller–Hinton broth

MIC: Minimum inhibitory concentration

MLST: Multilocus sequence typing

MRSA: Methicillin-resistant *Staphylococcus aureus*

MSSA: Methicillin-susceptible *Staphylococcus aureus*

OD_{600}: Optical density at a wavelength 600 nm

OS-MRSA: Oxacillin-susceptible MRSA

PBP2a: Penicillin-binding protein 2a

(p)ppGpp: Guanosine tetra- and penta-phosphate

qRT-PCR: Quantitative Reverse Transcription PCR

RNA-seq: RNA sequencing

RNA: Ribonucleic acid

RNAP: RNA polymerase

rRNA: ribosomal RNAs

SCC*mec*: staphylococcal cassette chromosome *mec*

SNPs: single nucleotide polymorphisms

ST: Sequence type of MLST

TSA: Tryptic soy agar

TSB: Tryptic soy broth

VISA: Vancomycin-Intermediate *Staphylococcus aureus*

2. Introduction

Staphylococcus aureus is an important bacterial pathogen that can cause life-threatening infections in both humans and animals (1, 2). A known feature of *S. aureus* is its evolutionary potential to develop antibiotic resistance under selection pressure via antibiotic treatment. Methicillin-resistant *S. aureus* (MRSA) is resistant to the entire class of β-lactam antibiotics, including penicillin, methicillin, and cefazolin (3). It was first recognized as a problematic pathogen in hospital settings, but it has subsequently emerged in community settings and livestock (3-5). MRSA infections remain a major concern in the clinical setting because they are more difficult to treat than infections involving other ordinary susceptible strains of *S. aureus*. β-lactam resistance in MRSA is primarily mediated by a non-native *mecA* gene encoding modified penicillin-binding protein 2a (PBP2a), which has an extremely low affinity for β-lactams, whereas PBP2a expression in MRSA is dependent on the presence of functional MecI/MecR1/MecR2 and BlaI/BlaR1 regulators in the *mec* and *bla* operons, respectively (6, 7). However, the level of β-lactam resistance does not always correlate with that of PBP2a expression (6-8).

Recently, oxacillin-susceptible *mecA*-positive *S. aureus* (OS-MRSA) strains have been increasingly reported worldwide in clinical isolates as well as in animals and

food (9-15). In clinical microbiology laboratories, an oxacillin minimum inhibitory concentration (MIC) ≥ 4 μg/mL or cefoxitin MIC ≥ 8 μg/mL is routinely used as a breakpoint for diagnosing MRSA, whereas the presence of *mecA* has been used as a marker for the genotypic identification of MRSA (16). Owing to its susceptibility to oxacillin, OS-MRSA might be misidentified as methicillin-susceptible *S. aureus* (MSSA) in routine clinical laboratories in which *mecA* detection is unavailable (17). In addition, despite being fundamentally susceptible to β-lactam treatment, OS-MRSA is prone to develop strong β-lactam resistance following antibiotic therapy due to its carriage of *mecA* (10-12, 14, 15). This ultimately leads to β-lactam treatment failure (18, 19). It is suggested that OS-MRSA possesses unknown regulatory mechanisms that contribute to the acquisition of β-lactam resistance. Therefore, this study aimed to unravel the genetic mechanism(s) responsible for the development of β-lactam resistance in OS-MRSA.

3. Materials and Methods

3-1. Bacterial strains and growth condition

A total of 43 OS-MRSA isolates from various clinical samples were collected from routine clinical laboratories in hospitals across Japan and Taiwan between 1998 and 2015 (Table 1 (20-22)). Mueller–Hinton broth (MHB; Becton Dickinson Co., Ltd., Sparks, MD, USA) and tryptic soy broth (TSB; Becton Dickinson) were used to culture *S. aureus*, whereas *Escherichia coli* was grown in Luria-Bertani (LB; Becton Dickinson) medium. In some experiments, antibiotics were added to the medium at the following concentrations: ampicillin (Nacalai Tesque, Inc., Kyoto, Japan) at 100 μg/mL for *E. coli*, tetracycline (Nacalai Tesque) at 5 μg/mL for *S. aureus*, and chloramphenicol (Nacalai Tesque) at 10 μg/mL for *S. aureus* and *E. coli*. For preservation, bacterial cells were cultivated on tryptic soy agar (TSA; Becton Dickinson) and incubated at 37°C upon receipt. A single colony was then selected and grown overnight in TSB at 37°C. The overnight culture was aliquoted and stored at −80°C in 50% glycerol (Wako Pure Chemical Industries, Ltd., Tokyo, Japan) until use.

Table 1: List of clinical OS-MRSA isolates used in this study

Strain name	Original name	Hospital	Isolated origin	Patient age (Year)	Isolation year	Isolation country	Oxacillin susceptibility test in a clinical laboratory			Reference
							Method	MIC (μg/mL)	Zone size (mm)	
JMUB217	JMUB217	A	pus	50	2015	Japan	NA [a]	NA	NA	This study
JMUB492	D2	B	sputum	61	2014	Japan	NA	NA	NA	Kanesaka I *et al* .[20]
JMUB1280	ISA19	C	skin	1	2005	Japan	Broth dilution	2	NA	Wada A *et al.* [21]
JMUB1281	ISA134	D	skin	2	2005	Japan	Broth dilution	2	NA	Wada A *et al.* [21]
JMUB1282	ISA180	E	sputum	72	2005	Japan	Broth dilution	2	NA	Wada A *et al.* [21]
JMUB1283	ISA193	D	skin	2	2005	Japan	Broth dilution	2	NA	Wada A *et al.* [21]
JMUB1284	ISA210	D	skin	5	2005	Japan	Broth dilution	2	NA	Wada A *et al.* [21]
JMUB1285	ISA218	F	pus	4	2005	Japan	Broth dilution	2	NA	Wada A *et al.* [21]
JMUB1286	ISA299	G	sputum	66	2005	Japan	Broth dilution	2	NA	Wada A *et al.* [21]
JMUB1288	ISA426	H	otorrhea	<1	2006	Japan	NA	NA	NA	This study
JMUB1289	ISA448	I	otorrhea	5	2006	Japan	Disk diffusion	NA	22	Wada A *et al.* [21]
JMUB1291	ISA503	J	pharynx	11	2006	Japan	NA	NA	NA	This study
JMUB1292	ISA509	I	otorrhea	2	2006	Japan	VITEK2	2	NA	Wada A *et al.* [21]
JMUB1293	ISA513	J	urine	NA	2006	Japan	VITEK2	2	NA	Wada A *et al.* [21]
JMUB1294	ISA537	K	otorrhea	64	2006	Japan	NA	NA	NA	This study
JMUB1295	ISA671	L	skin	2	2006	Japan	MicroScan	2	NA	Wada A *et al.* [21]
JMUB1296	ISA690	M	genital	23	2006	Japan	NA	NA	NA	This study
JMUB1297	ISA740	N	urine	33	2006	Japan	VITEK2	1	NA	Wada A *et al.* [21]
JMUB1298	ISA825	O	nasal cavity	9	2006	Japan	NA	NA	NA	This study
JMUB1299	ISA915	F	pus	3	2006	Japan	NA	NA	NA	This study
JMUB1300	ISA925	L	skin	4	2006	Japan	NA	NA	NA	This study
JMUB1301	ISA998	P	otorrhea	1	2006	Japan	NA	NA	NA	This study
JMUB1302	ISA1020	Q	otorrhea	3	2006	Japan	VITEK2	2	NA	Wada A *et al.* [21]
JMUB1303	ISA1091	H	otorrhea	7	2006	Japan	MIC2000	2	NA	Wada A *et al.* [21]
JMUB1304	SA06-50	R	skin	2	2005	Japan	NA	NA	NA	This study
JMUB1305	SA09-38	S	sputum	59	2005	Japan	NA	NA	NA	This study
JMUB1308	SA13-6	T	nasal mucosa	33	2006	Japan	NA	NA	NA	This study
JMUB1310	SA13-34	T	nasal mucosa	11	2006	Japan	NA	NA	NA	This study
JMUB1311	SA15-6	U	pharynx	6	2006	Japan	NA	NA	NA	This study
JMUB1312	RSA7520	D	skin	1	2005	Japan	NA	NA	NA	This study
JMUB1313	RSA7543	V	feces	72	2005	Japan	VITEK2	2	NA	Wada A *et al.* [21]
JMUB1314	RSA7544	W	otorrhea	43	2005	Japan	Broth dilution	2	NA	Wada A *et al.* [21]
JMUB1315	RSA7686	X	others	29	2005	Japan	VITEK2	0.5	NA	Wada A *et al.* [21]
JMUB1316	RSA7768	Y	nasal discharge	3	2005	Japan	NA	NA	NA	This study
JMUB1972	985414	Z	wound	NA	1998	Taiwan	NA	NA	NA	Chen FJ *et al* .[22]
JMUB1973	201698	Z	pus	NA	2000	Taiwan	Broth dilution	2	NA	Chen FJ *et al* .[22]
JMUB1974	2E03-095	Z	pus	53	2002	Taiwan	Broth dilution	2	NA	Chen FJ *et al* .[22]
JMUB1976	2E01-007	Z	pus	10	2002	Taiwan	Broth dilution	2	NA	Chen FJ *et al* .[22]
JMUB1977	4S06-084	Z	wound	56	2004	Taiwan	Broth dilution	2	NA	Chen FJ *et al* .[22]
JMUB1978	4C09-188	Z	pus	<1	2004	Taiwan	Broth dilution	2	NA	Chen FJ *et al* .[22]
JMUB1979	6E03-015	Z	pus	23	2006	Taiwan	Broth dilution	2	NA	Chen FJ *et al* .[22]
JMUB1980	6N21-147	Z	wound	34	2006	Taiwan	Broth dilution	2	NA	Chen FJ *et al* .[22]
JMUB1981	8S05-106	Z	pus	47	2008	Taiwan	Broth dilution	2	NA	Chen FJ *et al* .[22]

[a]NA: Not available

3-2. Antibiotic susceptibility test

Oxacillin and cefoxitin MICs were determined using the E-test method according to Clinical and Laboratory Standards Institute (CLSI) guidelines (16). Briefly, overnight cultures of *S. aureus* strains grown in 4 mL of MHB at 37°C were adjusted to 0.5 McFarland turbidity (approximately 1×10^8–2×10^8 CFU/mL) and spread on Mueller–Hinton agar (MHA; Becton Dickinson) plates. The E-test gradient strip (bioMérieux SA, Marcy l'Étoile, France) was then placed on the bacterial lawn. The MIC was determined after incubation at 37°C for 24 h. The isolates with oxacillin MIC ≤ 2 μg/mL or cefoxitin MIC ≤ 4 μg/mL were considered oxacillin- and cefoxitin-susceptible, respectively.

3-3. *mecA* detection via PCR

DNA was extracted from OS-MRSA isolates grown overnight on TSA plates using MightyPrep reagent (Takara Bio Inc., Shiga, Japan) in accordance with the manufacturer's instructions. PCR was then performed on the extracted DNA using Quick Taq® HS DyeMix (Toyobo Co., Ltd., Osaka, Japan). A primer pair (*mecA*-F and *mecA*-R, Table 2) was used to amplify a 519-bp region of *mecA*. The thermal cycling conditions included initial denaturation at 94°C for 2 min followed by 30 cycles of 94°C for 30 s,

55°C for 30 s, and 68°C for 1 min. Finally, the amplified products were electrophoresed on 1% agarose gel, stained with ethidium bromide, and visualized using AE-6933FXES Printgraph (Atto Co., Tokyo, Japan).

Table 2: List of primers used to construct mutants

Primer name	Primer sequence (5' to 3')	Purpose
mecA-F	TGTCCGTAACCTGAATCAGC	Detection of *mecA* by PCR
mecA-R	TGCTATCCACCCTCAAACAG	Detection of *mecA* by PCR
mecA-F-qRT-PCR	GAAGTAGAAATGACTGAACGTCCG	Detection of *mecA* by qRT-PCR
mecA-R-qRT-PCR	CGTTGCGATCAATGTTACCGTAG	Detection of *mecA* by qRT-PCR
rho-F-qRT-PCR	GAAGCTGCTGAAGTCG	Detection of *rho* by qRT-PCR
rho-R-qRT-PCR	GAATGCTTTGGTTTGTGTAA	Detection of *rho* by qRT-PCR
SacI-mecAKO-UP-2	TTTTgagctcGACGGTGATCTTGCTCAATGAG	Construction of *mecA* knockout mutant
PstI-mecAKO-UP	TTTTctgcagTAGTAAACACTGAAGATGCC	Construction of *mecA* knockout mutant
mecA_fPCR_UP	CGGATTGCTTCACTGTTTTGCAATATCCTCCTTATATAAG	Construction of *mecA* knockout mutant
mecA_fPCR_DN	CTTATATAAGGAGGATATTGCAAAACAGTGAAGCAATCCG	Construction of *mecA* knockout mutant
BlaI-1-1	aaaaGGATCCTTGGCGTGCCATTATCATAA	Construction of *blaI*-1 knockout mutant
BlaI-2-1	aaaaGGATCCAAATACTTTTAAAAAATAAG	Construction of *blaI*-2 knockout mutant
BlaI-1,2-2	aaaaCTGCAGCTGATTATAATTATAAAAAG	Construction of *blaI*-1/2 knockout mutant
BlaI-1,2-3	TTAAAACACCCATTTCTTTT	Construction of *blaI*-1/2 knockout mutant
BlaI-1-4	TTAGAGATATGTTTCAAACA	Construction of *blaI*-1 knockout mutant
BlaI-2-4	ATAGATATAGGGTTCAAACA	Construction of *blaI*-2 knockout mutant
SmaI-mecAcomp-F-pKAT	TTTTcccgGGATATTTTATATAGAGCATTCTC	Construction of *mecA* complementation mutant
SalI-mecAcomp-R-pKAT	TTTTgtcgacTTAAGGGAGAAGTAACAGCAC	Construction of *mecA* complementation mutant

3-4. Isolation of mutants with reduced susceptibility to oxacillin from parental OS-MRSA strains

To isolate mutants with reduced oxacillin susceptibility, all 43 OS-MRSA parental strains were exposed to oxacillin according to the E-test method as described for susceptibility testing. Briefly, the oxacillin E-test was performed using OS-MRSA strains inoculated onto MHA plates. A single colony growing inside the inhibition zone after 24–48 h of incubation was randomly picked and sub-cultured in TSB for 24 h at 37°C. The overnight culture was then serially diluted 10-fold with 0.9% NaCl and spread onto a TSA plate. A single colony growing on the TSA plate was again randomly selected and inoculated into TSB for preservation in 50% glycerol at −80°C. The oxacillin susceptibility of the stocked cells was determined again using the E-test method to discriminate mutant colonies from persister colonies. The cells exhibiting higher oxacillin MICs were selected as oxacillin-reduced susceptibility mutants, which were then used for subsequent analysis.

3-5. Whole-genome sequencing

Genomic DNA was extracted from OS-MRSA and its mutants using the phenol-chloroform method and purified using a QIAamp DNA mini kit (Qiagen, Hilden,

Germany) following previously developed methods (23). The genomic sequences of parental strains were determined via mate-pair sequencing as previously described (23, 24). Briefly, a mate-pair library was prepared using a Nextera mate-pair library prep kit (Illumina, Inc., San Diego, CA, USA), and sequencing was performed using an Illumina MiSeq platform with the MiSeq reagent kit version 3 (Illumina). The mate-paired reads of OS-MRSA were trimmed using the FASTQ toolkit version 2.2.0 to generate high-quality reads and assembled using Velvet Assembler version 1.2.10 to construct genome scaffolds. The generated genomic sequences were finally annotated using Microbial Genome Annotation Pipeline (http://www.migap.org/). Meanwhile, the genomic sequences of *in vitro*-selected mutants with reduced oxacillin susceptibility were determined by sequencing paired-end reads as previously described (25). The paired-end library was prepared using a Nextera XT library prep kit and sequenced using the Illumina MiSeq platform with the MiSeq reagent kit version 3. The paired-end reads of each mutant were mapped against the genomic sequences of their corresponding parental OS-MRSA strains, and mutations were detected using CLC Genomics Workbench version 9 (CLCbio, Qiagen, Valencia, CA, USA). Mutations identified in each mutant were verified by Sanger sequencing using the Applied Biosystems 3130xl genetic analyzer (Thermo Fisher Scientific, MA, USA).

3-6. Construction of the phylogenetic tree

To construct the OS-MRSA phylogenetic tree, kSNP3 (26), available at https://sourceforge.net/projects/ksnp/, was first used to identify single nucleotide polymorphisms (SNPs) in the whole-genome sequencing data of OS-MRSA strains. The k-mer size was set to an optimum length of 13 as estimated by Kchooser for extracting SNPs from the sequence data. A maximum parsimony tree was then constructed using the majority of the SNPs present in at least 75% of the genomes. The generated phylogenetic tree was visualized using FigTree ver.1.4.3 (tree.bio.ed.ac.uk/software/figtree/).

3-7. Growth curve analysis

The bacterial doubling time was determined as described previously (27). Briefly, overnight cultures of parental OS-MRSA strains and the laboratory-selected mutants were adjusted to an OD_{600} of 0.2 in MHB. Then, aliquots of 100 μL were inoculated into 10 mL of MHB (final concentration of 1×10^5 CFU/mL), and the cultures were grown at 37°C with agitation at 25 rpm in an automatic temperature gradient rocking incubator (model TVS126MB; Advantec, Tokyo, Japan). The cell densities at OD_{600} were

measured every 5 min for 12 h, and the bacterial growth curve was generated by plotting the measured ODs against time. The doubling time was determined by fitting the growth curve to an exponential equation. Bacterial growth was measured using at least three independent experiments.

3-8. Determination of cellular ATP level

The parental OS-MRSA strains and the laboratory-selected mutants were cultured overnight in MHB at 37°C with agitation at 150 rpm. The overnight cultures were adjusted to an OD_{600} of 0.2 in MHB, and 100 μL of the OD-adjusted culture were inoculated into 10 mL of MHB. The cultures were grown at 37°C with agitation at 25 rpm in an automatic temperature gradient rocking incubator. One microliter of each mid-exponential phase culture (OD_{600} = 0.5) was then transferred to a fresh 1.5-mL tube and immediately centrifuged at 15,000 rpm for 1 min at 4°C to pellet cells. After centrifugation, the cell pellet was stored immediately at −80°C until analysis. To determine cellular ATP levels, a BacTiter-Glo™ Microbial Cell Viability Assay kit (Promega, WI, USA) was used according to the manufacturer's instructions. Briefly, the cell pellet was resuspended in 1 mL of MHB, and 25 μL of the cell suspension were mixed with an equal volume of BacTiter-Glo Reagent in a 384-well opaque plate (Iwaki,

Tokyo, Japan) and incubated at room temperature for 5 min. The luminescence was then read on an EnVision 2104 Multilabel Reader (Perkin Elmer, Waltham, MA, USA). The ATP concentration was determined with reference to an ATP standard curve prepared from ATP disodium salt hydrate (A2383, Merck KGaA, Darmstadt, Germany). ATP disodium salt was dissolved in distilled water, yielding 1 μM ATP standard solutions. Serial 10-fold dilutions of the ATP standard solution were created using MHB to prepare diluted standards that were then used to generate the standard curve. The cellular ATP concentration of each sample was presented as the mean of three independent experiments performed using three biological replicates.

3-9. RNA extraction

Overnight cultures of the parental OS-MRSA strains and the laboratory-selected mutants were adjusted to an OD_{600} of 0.4. The OD-adjusted cultures were then diluted 1:100 in 1 or 10 mL of MHB for qRT-PCR and RNA-seq, respectively. Each culture was grown to the early log-phase (OD_{600} = 0.3) before treatment with a final concentration of 0.1 μg/mL oxacillin or equal volume of distilled water (control) for 1 h (qRT-PCR) or until OD_{600} = 0.6 (RNA-seq). After oxacillin treatment, the bacterial cells were harvested

by centrifugation at 15,000 rpm for 1 min at 4°C (qRT-PCR) or at 8,000 rpm for 5 min at 4°C (RNA-seq). Pelleted cells were resuspended in 600 µL (qRT-PCR) or 6 mL (RNA-seq) of TE buffer (10 mM Tris-HCl and 10 mM EDTA, pH 8.0) and lysed with 25 (qRT-PCR) or 30 µg (RNA-seq) of lysostaphin (Merck KGaA) by incubating the mixtures at 37°C for less than 5 min. Total RNA was then extracted using acidic-phenol saturated with 20 mM sodium acetate (pH 4.8) and chloroform and enriched via ethanol precipitation. Contaminating DNA was removed from the total RNA preparations by incubating the solutions with 2 (qRT-PCR) or 20 units (RNA-seq) of RNase-free DNase I (F. Hoffmann-La Roche Ltd, Basel, Switzerland) at 37°C for 30 min. Total RNA was finally purified using acidic-phenol/chloroform and eluted in RNase-free water.

3-10. Determination of *mecA* expression level by qRT-PCR

The extracted total RNA (100 ng per sample) was reverse-transcribed into complementary DNA (cDNA) using a PrimeScript™ 1st Strand cDNA Synthesis Kit (Takara Bio). qRT-PCR was performed using TB Green™ *Premix Ex Taq*™ (Tli RNaseH Plus, Takara Bio) on the Mx3005P™ Real-Time PCR instrument (Stratagene, La Jolla, CA, USA). A primer set (*mecA*-F-qRT-PCR and *mecA*-R-qRT-PCR, Table 2) was used

to amplify the 162-bp *mecA* sequence, whereas the 163-bp housekeeping gene *rho* was amplified using designated primers (*rho*-F-qRT-PCR and *rho*-R-qRT-PCR, Table 2) and used as the reference gene for normalization during gene expression analysis. The thermal cycling conditions included initial denaturation at 95°C for 30 s followed by 40 cycles of 95°C for 5 s and 60°C for 30 s.

3-11. RNA-sequencing analysis

To perform RNA-seq analysis, ribosomal RNAs (rRNAs) in total RNA preparations of the JMUB217 strain and its mutant derivatives were first depleted using a Ribo-Zero rRNA Removal Kit (Bacteria) from Illumina. Double-stranded cDNA was then synthesized using a PrimeScript Double Strand cDNA Synthesis Kit (Takara Bio). The generated cDNA served as the template for constructing the paired-end library using a Nextera XT library prep kit, and the library was subsequently sequenced using the Illumina MiSeq platform and the MiSeq reagent kit version 3. RNA-seq analysis was performed using CLC Genomics Workbench version 9, and the RNA-seq reads were aligned to the reference genomes of the parental strain JMUB217. Gene expression was normalized by calculating the reads per kilobase per million mapped reads, and

differentially expressed genes were identified using Baggerly's test (β-binomial test) with false discovery rate correction. Genes with adjusted $p < 0.05$ were considered to be significantly differentially expressed.

3-12. Construction of *mecA* and *blaI* knockout mutants

To construct *mecA* and *blaI*-knockout mutants of the JMUB217 strain, the pKFT markerless gene deletion system was used as previously described (28). Briefly, to delete *mecA*, 1-kb upstream and downstream flanking sequences of the target gene were amplified by PCR using the primer sets SacI-mecAKO-UP-2/mecA_fPCR_UP and PstI-mecAKO-UP/mecA_fPCR_DN (Table 2), respectively, and KOD FX Neo (Toyobo). Then, second-round PCR was performed using the first-round PCR products as templates with the primer set SacI-mecAKO-UP-2/PstI-mecAKO-UP. The second-round PCR products and pKFT were digested with the restriction enzymes *Pst*I and *Sac*I (Takara Bio) and ligated using Ligation high ver. 2 (Toyobo), generating the plasmid pmecAKO. pmecAKO was transformed into *E. coli* DH5α, and the transformed cells were plated on LB agar with 100 μg/mL ampicillin. Concerning the generation of *blaI*-knockout mutants, DNA fragments containing *blaI*-1 (locus tag: JMUB217_1395) or *blaI*-2 (locus tag: JMUB217_2048) were first amplified with the primer sets BlaI-1-

1/BlaI-1,2-2 and BlaI-2-1/BlaI-1,2-2 (Table 2), respectively. The PCR fragments and pKFT were then digested using the restriction enzymes *Bam*HI and *Pst*I (Takara Bio) and ligated using Ligation high ver. 2. The ligated DNA fragments were independently transformed into *E. coli* DH5α, and the transformed cells were plated on LB agar with 100 μg/mL ampicillin. The plasmids were extracted, and second-round PCR was conducted using the primer set BlaI-1,2-3/BlaI-1-4 for *blaI*-1 knockout strains and BlaI-1,2-3/BlaI-2-4 for *blaI*-2 knockout strains. The self-ligated PCR fragments (pblaI-1KO and pblaI-2KO) were again individually transformed into *E. coli* DH5α, and transformed cells were plated on LB agar with 100 μg/mL ampicillin. Afterward, all three plasmids (pmecAKO, pblaI-1KO, and pblaI-2KO) were extracted from the *E. coli* DH5α transformants and transformed into *E. coli* BL21. The plasmids extracted from *E. coli* BL21 were subsequently electroporated into *S. aureus* JMUB217 and mutants with reduced oxacillin susceptibility as previously described (29), and the cells were cultured on TSA with 5 μg/mL tetracycline at 30°C. An isolated colony was then grown overnight in 4 mL of TSB containing 5 μg/mL tetracycline at 30°C. Single crossover was performed by growing the overnight culture on TSA with 5 μg/mL tetracycline at 43°C. Then, double crossover was performed by incubating the single crossover mutant

on TSA at 30°C. The double crossover event was confirmed by PCR and Sanger sequencing.

3-13. Complementation of *mecA*

To generate a *mecA*-complemented mutant, a DNA fragment containing wild-type *mecA* from strain JMUB217 was amplified using the primers *Sma*I-mecAcomp-F-pKAT and *Sal*I-mecAcomp-R-pKAT (Table 2). The PCR fragment and pKAT were digested with *Sma*I and *Sal*I (Takara Bio) and ligated using Ligation high ver. 2. The ligated DNA fragment was transformed into *E. coli* DH5α, and the transformed cells were plated on LB agar with 10 μg/mL chloramphenicol. Finally, the complementation plasmid was extracted and electroporated into the JMUB217 strain (29).

3-14. Statistical analysis

All statistical analyses were performed using Prism 8 (GraphPad Software, San Diego, CA, USA). The correlations between variables were calculated using the non-parametric Spearman's correlation coefficient (r_s), with $p < 0.05$ denoting statistical significance.

4. Results

4-1. Characterization of clinical OS-MRSA isolates

A total of 43 OS-MRSA isolates recovered from various clinical specimens collected from Japan and Taiwan were included in this study (Table 1). The characteristics of OS-MRSA were re-confirmed via determination of their oxacillin susceptibility and the presence of *mecA* (Table 3). Our results revealed that all studied strains maintained the typical characteristics of OS-MRSA, including *mecA* positivity, but they were susceptible to oxacillin with MICs ranging from 0.125 to 2 μg/mL. According to CLSI, cefoxitin can also be used to detect MRSA. Thus, cefoxitin susceptibility testing was conducted to investigate whether there was discrepancy between oxacillin and cefoxitin susceptibility among the OS-MRSA isolates. The cefoxitin MICs for all OS-MRSA isolates ranged 1.5–12 μg/mL, with 24 of the 43 OS-MRSA isolates (56%) exhibiting susceptibility to cefoxitin.

Table 3: Characteristics of the 43 clinical OS-MRSA isolates

		MIC (μg/mL)		MLST								SCC *mec* typing			
Clade [a]	Strain	Oxacillin	Cefoxitin	*arcC*	*aroE*	*glpF*	*gmk*	*pta*	*tpi*	*yqiL*	ST [b]	SCC *mec* type	*mecR1*	*mecI*	*mecA* [e]
1	JMUB1297	0.25	1.5	3	3	1	1	4	4	3	8	IVa	+	ND [d]	+
	JMUB1282	0.125	2	3	3	1	1	4	4	3	8	IVa	+	ND	+
	JMUB1315	0.25	3	3	3	1	42	4	4	3	1516	IVc	+	ND	+
2	JMUB492	2	12	1	1	1	1	1	1	1	1	IVa	+	ND	+
3	JMUB217	0.75	4	1	1	1	1	22	1	1	772	V	+	ND	+
4	JMUB1293	0.125	1.5	1	4	1	4	12	1	10	5	II	+	+	+
5	JMUB1308	0.5	4	1	26	28	18	18	33	50	89	IVa	+	ND	+
	JMUB1311	0.19	4	1	26	28	18	18	33	50	89	IVa	+	ND	+
	JMUB1291	1.5	6	1	26	28	18	18	33	50	89	IVa	+	ND	+
	JMUB1305	0.5	4	1	26	28	18	18	54	50	91	IVa	+	ND	+
	JMUB1285	0.5	6	1	26	28	18	18	33	50	89	V	+	ND	+
	JMUB1289	0.5	6	1	26	28	18	18	33	50	89	V	+	ND	+
	JMUB1284	0.5	4	1	26	28	18	18	33	50	89	V	+	ND	+
	JMUB1283	0.38	4	1	26	28	18	18	33	50	89	V	+	ND	+
	JMUB1304	0.75	4	1	26	28	18	18	33	50	89	V	+	ND	+
	JMUB1301	1	6	1	26	28	18	18	33	50	89	V	+	ND	+
6	JMUB1973	0.75	6	19	23	15	2	19	20	15	59	V	+	ND	+
	JMUB1981	1	6	19	23	15	2	19	20	15	59	V	+	ND	+
	JMUB1980	0.5	4	19	23	15	2	19	20	15	59	V	+	ND	+
	JMUB1976	1.5	6	19	23	15	2	19	20	15	59	V	+	ND	+
	JMUB1978	0.75	6	19	23	15	2	19	20	15	59	V	+	ND	+
	JMUB1972	1	6	19	23	15	2	19	20	15	59	V	+	ND	+
	JMUB1974	0.25	1.5	19	23	15	2	19	20	15	59	V	+	ND	+
	JMUB1977	0.75	6	19	23	15	48	19	20	15	338	V	+	ND	+
	JMUB1979	0.5	8	19	23	15	2	19	20	15	59	IVa	+	ND	+
7	JMUB1298	0.75	8	6	5	6	2	7	14	5	121	V	+	ND	+
	JMUB1303	0.25	4	6	5	6	2	7	14	5	121	V	+	ND	+
	JMUB1281	1	6	6	5	6	2	7	14	5	121	V	+	ND	+
	JMUB1286	1	6	6	5	6	2	7	14	5	121	V	+	ND	+
	JMUB1300	0.19	1.5	6	5	6	2	7	14	5	121	V	+	ND	+
	JMUB1314	0.75	6	6	5	6	2	7	14	5	121	V	+	ND	+
	JMUB1312	1	4	6	5	6	2	7	14	5	121	V	+	ND	+
	JMUB1313	0.75	2	6	5	6	2	7	14	5	121	V	+	ND	+
	JMUB1288	0.5	2	6	5	6	2	7	14	5	121	V	+	ND	+
	JMUB1280	0.5	4	6	5	6	2	7	14	5	121	V	+	ND	+
	JMUB1295	0.19	3	6	5	6	2	7	14	5	121	V	+	ND	+
	JMUB1299	0.25	4	6	5	6	2	7	14	5	121	V	+	ND	+
	JMUB1316	0.75	6	6	5	6	2	7	14	5	121	V	+	ND	+
	JMUB1294	0.38	6	6	5	6	2	7	14	5	121	V	+	ND	+
	JMUB1296	0.5	4	6	5	6	2	7	14	5	121	V	+	ND	+
	JMUB1292	0.75	4	6	5	6	2	7	14	5	121	V	+	ND	+
	JMUB1310	0.5	4	73	5	6	2	7	14	5	NT [c]	V	+	ND	+
	JMUB1302	1	6	73	5	6	2	7	14	5	NT	V	+	ND	+

[a]Clade classified from a particular branch on the phylogenetic tree

[b]Sequence type

[c]Non-typeable

[d]Not detected

[e]Detected by PCR and whole genome sequencing data

4-2. Genome analysis of the clinical OS-MRSA isolates

To determine the genetic background of the strains used in this study, the whole-genome sequences of the 43 clinical OS-MRSA isolates were determined, and their phylogenetic relationships were analyzed by constructing a phylogenetic tree using kSNP3 (Fig 1). The phylogenetic tree revealed extensive genomic diversity among the isolates, which could be classified into seven main phylogenetic clades. In addition, these isolates could also be grouped into 11 MLST types (ST1, ST5, ST8, ST59, ST89, ST91, ST121, ST338, ST772, ST1516, and ST-non-typeable [STNT]), and they carried four different types of SCC*mec* types (II, IVa, IVc, and V). The majority of OS-MRSA isolates were belonged to ST121-SCC*mec* type V (16 strains, 37%), followed by ST59-SCC*mec* type V (seven strains, 16%), ST89-SCC*mec* type V (six strains, 14%), ST89-SCC*mec* type IVa (three strains, 7.0%), ST8-SCC*mec* type IVa (two strains, 4.7%), STNT-SCC*mec* type V (two strains, 4.7%). In addition, seven singletons (ST1-SCC*mec* type IVa, ST5-SCC*mec* type II, ST59-SCC*mec* type IVa, ST91-SCC*mec* type IVa, ST338-SCC*mec* type V, ST772-SCC*mec* type V, and ST1516-SCC*mec* type IVc), each of which comprised 2.3% of all strains, were identified. SCC*mec* types V (33 strains, 77%) and IVa (eight strains, 19%) were predominant among the OS-MRSA isolates, whereas only one isolate harbored each of SCC*mec* type II and IVc, respectively. Moreover, whole-

genome sequencing demonstrated that 34 of the 43 (79%) OS-MRSA isolates carried a complete *bla* operon (Table 4), which could be classified into two genotypes, namely *bla* operon-1 and *bla* operon-2, based on the nucleotide sequences. These two operons shared nucleotide identities of 94% for *blaZ*, 92% for *blaR1*, and 94% for *blaI*. Twelve strains (28%) carried *bla* operon-1, and all but one (JMUB217) *bla* operon-1 was located on plasmids. Meanwhile, 23 isolates (53%) carried intact *bla* operon-2 in their chromosomes. JMUB217 carried both *bla* operons in its chromosome. An incomplete *bla* operon-2 lacking *blaZ* but having intact *blaR1* and *blaI* was present in isolates JMUB1301 and JMUB1313. The absence of *blaZ* in the *bla* operons of these isolates was confirmed by PCR (data not shown). Lastly, seven isolates (16%) lacked a *bla* operon.

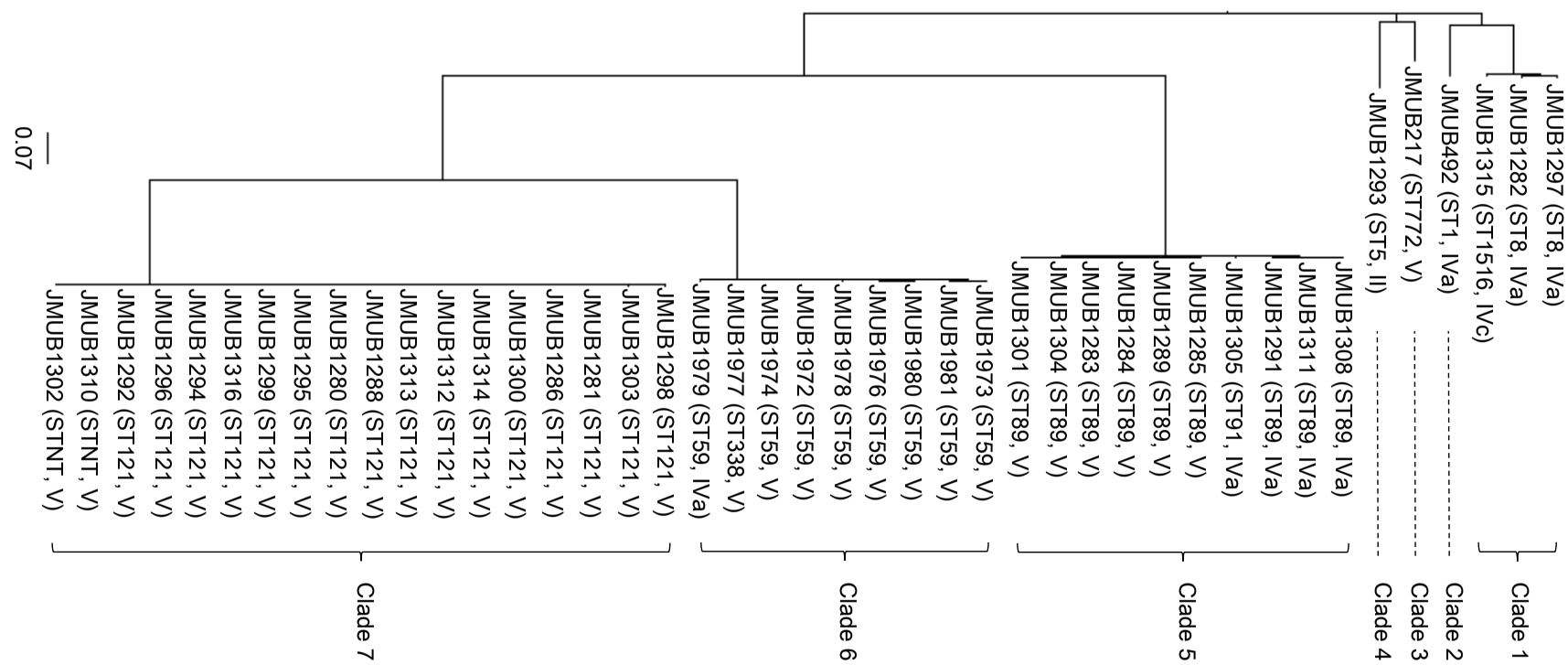

Fig 1: Phylogenetic relationships among clinical oxacillin-susceptible methicillin-resistant *S. aureus* (OS-MRSA) strains.

A maximum parsimony tree of 43 OS-MRSA isolates was generated from the majority of single nucleotide polymorphisms in the core genome and edited with FigTree ver.1.4.3. The strain name is followed by the sequence type (ST) of MLST and SCC*mec* type. OS-MRSA isolates were classified into seven main clades (clades 1–7).

Table 4: *bla* operon in OS-MRSA

			bla operon-1				*bla* operon-2			
Strain	ST[a]	SCC*mec* type	*blaR1*	*blaI*	*blaZ*	location	*blaR1*	*blaI*	*blaZ*	location
JMUB1297	8	IVa	ND[c]	ND	ND	-	ND	ND	ND	-
JMUB1282	8	IVa	+	+	+	Plasmid	ND	ND	ND	-
JMUB1315	1516	IVc	ND	ND	ND	-	ND	ND	ND	-
JMUB492	1	IVa	+	+	+	Plasmid	ND	ND	ND	-
JMUB217	772	V	+	+	+	Chromosome	+	+	+	Chromosome
JMUB1293	5	II	ND	ND	ND	-	ND	ND	ND	-
JMUB1308	89	IVa	ND	ND	ND	-	+	+	+	Chromosome
JMUB1311	89	IVa	ND	ND	ND	-	+	+	+	Chromosome
JMUB1291	89	IVa	ND	ND	ND	-	+	+	+	Chromosome
JMUB1305	91	IVa	ND	ND	ND	-	+	+	+	Chromosome
JMUB1285	89	V	ND	ND	ND	-	+	+	+	Chromosome
JMUB1289	89	V	ND	ND	ND	-	+	+	+	Chromosome
JMUB1284	89	V	ND	ND	ND	-	+	+	+	Chromosome
JMUB1283	89	V	ND	ND	ND	-	+	+	+	Chromosome
JMUB1304	89	V	ND	ND	ND	-	+	+	+	Chromosome
JMUB1301	89	V	ND	ND	ND	-	+	+	ND	Chromosome
JMUB1973	59	V	+	+	+	Plasmid	ND	ND	ND	-
JMUB1981	59	V	+	+	+	Plasmid	ND	ND	ND	-
JMUB1980	59	V	+	+	+	Plasmid	ND	ND	ND	-
JMUB1976	59	V	+	+	+	Plasmid	ND	ND	ND	-
JMUB1978	59	V	+	+	+	Plasmid	ND	ND	ND	-
JMUB1972	59	V	+	+	+	Plasmid	ND	ND	ND	-
JMUB1974	59	V	+	+	+	Plasmid	ND	ND	ND	-
JMUB1977	338	V	+	+	+	Plasmid	ND	ND	ND	-
JMUB1979	59	IVa	+	+	+	Plasmid	ND	ND	ND	-
JMUB1298	121	V	ND	ND	ND	-	ND	ND	ND	-
JMUB1303	121	V	ND	ND	ND	-	+	+	+	Chromosome
JMUB1281	121	V	ND	ND	ND	-	+	+	+	Chromosome
JMUB1286	121	V	ND	ND	ND	-	+	+	+	Chromosome
JMUB1300	121	V	ND	ND	ND	-	+	+	+	Chromosome
JMUB1314	121	V	ND	ND	ND	-	+	+	+	Chromosome
JMUB1312	121	V	ND	ND	ND	-	+	+	+	Chromosome
JMUB1313	121	V	ND	ND	ND	-	+	+	ND	Chromosome
JMUB1288	121	V	ND	ND	ND	-	+	+	+	Chromosome
JMUB1280	121	V	ND	ND	ND	-	+	+	+	Chromosome
JMUB1295	121	V	ND	ND	ND	-	+	+	+	Chromosome
JMUB1299	121	V	ND	ND	ND	-	+	+	+	Chromosome
JMUB1316	121	V	ND	ND	ND	-	ND	ND	ND	-
JMUB1294	121	V	ND	ND	ND	-	ND	ND	ND	-
JMUB1296	121	V	ND	ND	ND	-	ND	ND	ND	-
JMUB1292	121	V	ND	ND	ND	-	+	+	+	Chromosome
JMUB1310	NT[b]	V	ND	ND	ND	-	+	+	+	Chromosome
JMUB1302	NT	V	ND	ND	ND	-	+	+	+	Chromosome

[a]Sequence type of MLST

[b]Non-typable

[c]Not detected

4-3. Identification of mutations associated with reduced susceptibility to oxacillin in OS-MRSA

To elucidate the pathway(s) leading to the acquisition of β-lactam resistance in OS-MRSA, laboratory-derived mutants with reduced susceptibility to oxacillin were obtained from the parental OS-MRSA strains via single-step exposure to oxacillin. Although resistant colonies growing inside the inhibition zone were generated from all 43 parental strains, not all selected colonies displayed increased oxacillin MICs after single-colony purification. Re-determination of the oxacillin susceptibility of the isolated colonies identified 100 *in vitro*-selected mutants with increased MICs to oxacillin (range, 1–256 μg/mL) from 26 parental OS-MRSA strains representing all seven phylogenetic clades (Fig 1), with 86 mutants exhibiting MICs exceeding 4 μg/mL (Table 5). The comparative genomic analysis of the 100 mutants and their corresponding parental OS-MRSA isolates identified a total of 141 mutations in the selected OS-MRSA mutants (Table 5). The identified mutations were verified via Sanger sequencing (data not shown). Seventy mutants had only one mutation in a single gene, four mutants carried two mutations in a single gene, and 26 mutants had mutations in multiple loci including intergenic regions. Ninety-six mutants carried at least one nonsynonymous or frameshift mutation in their genomes, suggesting that the mutations may affect cellular metabolism. Only four mutants (JMUB1283-3, JMUB1972-1, JMUB1281-7, and JMUB1310-6) had

silent mutations (HP7^{A450G}, HP10^{G651A}, *guaA*^{G1158A}, and *tilS*^{T1287G}; Table 5). Among the 129 mutations identified in coding sequences, 98 (76%), 13 (10%), 10 (7.8%), and 8 (6.2%) were missense, nonsense, frameshift, and synonymous mutations, respectively. Of the 129 mutations, 121 nonsynonymous mutations were identified in 46 ORFs (Table 5 and Fig 2). However, no mutation was identified in *mec* or *bla* operons. As shown in Table 5, 129 mutations identified among 100 mutants were located in 46 genes. The mutated genes could be classified into 12 functional categories: (i) DNA/RNA polymerase, *rpoC* (22 mutations), *rpoB* (20 mutations), and *dnaE* (one mutation); (ii) purine biosynthesis, *guaA* (nine mutations), *rsh* (four mutations), *hprT* (three mutations), and *relQ* (one mutation); (iii) protein quality control, *clpP* (six mutations), *clpX* (three mutations), *ftsH* (one mutation), and *yjbH* (one mutation); (iv) membrane protein associated with glycopeptide resistance, *mprF* (four mutations), *tcaA* (one mutation), and *vraT* (three mutations); (v) glycolysis, *fruB* (five mutations), *fbaA* (two mutations), *ptsI* (one mutation), and *pykA* (one mutation); (vi) pentose phosphate pathway, *rpiA* (one mutation) and *prs* (five mutations); (vii) tRNA synthesis, *thrS* (one mutation), *tilS* (one mutation), *gltX* (one mutation), and *lysS* (one mutation); (viii) folate biosynthesis, *folC* (one mutation); (ix) peptidoglycan biosynthesis, *sgtB* (one mutation); (x) transcriptional regulation, *mraZ* (one mutation); (xi) extracellular matrix protein, *ebhA* (one mutation); and (xii) unknown function, HP1-HP18 (27 mutations). Conversely, the 12 mutations

identified in eight intergenic regions were located between SA0499 and *rpoB*, between *sgtB* and SA1692, between E8M03_00305 and *hsdR*, between SA2092 and *ssaA2*, between SAS044 and SA1196, between *norB* and *ebhA*, between *tnp* and *proP*, and between SA1447 and *alas*, respectively. These results clearly demonstrated that the mutations associated with reduced oxacillin susceptibility in OS-MRSA occurred in a wide variety of types and locations.

Table 5: List of mutations in oxacillin-reduced susceptibility mutants

Mutant strain	Oxacillin MIC (μg/mL)	Mutated gene	Locus tag in N315	Nucleotide change	Amino acid change	Product	Functional category
JMUB1315-1	3	*rpoB*	SA0500	G2780A	G927D	RNA polymerase beta chain	DNA/RNA polymerase
JMUB492-1	12	*mprF*	SA1193	A2396G	F799S	Phosphatidylglycerol lysyltransferase	Membrane protein associated with glycopeptide resistance
JMUB492-2	12	*mprF*	SA1193	A2396G	F799S	Phosphatidylglycerol lysyltransferase	Membrane protein associated with glycopeptide resistance
JMUB492-3	16	*mprF*	SA1193	A2396G	F799S	Phosphatidylglycerol lysyltransferase	Membrane protein associated with glycopeptide resistance
JMUB492-4	16	*mprF*	SA1193	A2396G	F799S	Phosphatidylglycerol lysyltransferase	Membrane protein associated with glycopeptide resistance
JMUB217-1	1.5	*fruB*	SA0654	G106Del	G39fs	Fructose 1-phosphate kinase	Glycolysis
		HP1	SA0297	AAAG563-6Del	K191fs	Hypothetical protein, similar to ABC transporter (ATP-binding protein)	Unknown function
		HP2	SA0705	C136T	Q46*	Hypothetical protein, similar to comF operon protein 1	Unknown function
JMUB217-2	3	*tcaA*	SA2146	T643C	N215D	Membrane-associated protein TcaA	Membrane protein associated with glycopeptide resistance
		intergenic-1	between SA0499 and SA500 (*rpoB*)	T41A	-	between genes encoding conserved hypothetical protein and RNA polymerase beta chain	Intergenic region
JMUB217-3	3	*rpoB*	SA0500	T2255A	M752K	RNA polymerase beta chain	DNA/RNA polymerase
JMUB217-4	4	*fruB*	SA0654	G106Del	G39fs	Fructose 1-phosphate kinase	Glycolysis
JMUB217-5	4	*rpoB*	SA0500	C1441G	H481D	RNA polymerase beta chain	DNA/RNA polymerase
JMUB217-6	4	*relQ*	SA0864	C536T	A179V	GTP pyrophosphokinase	Purine biosynthesis pathway
JMUB217-7	4	*rpiA*	SA2127	C191A	A64E	Ribose-5-phosphate isomerase A	Pentose phosphate pathway
JMUB217-8	4	*rpoC*	SA0501	C2267A	A756D	RNA polymerase beta-prime chain	DNA/RNA polymerase
JMUB217-9	4	*hprT*	SA0468	G466A	G156S	Hypoxanthine-guanine phosphoribosyltransferase	Purine biosynthesis pathway
		HP2	SA0705	C136T	Q46*	Hypothetical protein, similar to comF operon protein 1	Unknown function
JMUB217-10	4	*fbaA*	SA1927	G62A	G21D	Fructose-bisphosphate aldolase	Glycolysis

Table 5-continued

Mutant strain	Oxacillin MIC (μg/mL)	Mutated gene	Locus tag in N315	Nucleotide change	Amino acid change	Product	Functional category
JMUB217-11	4	*rpoC*	SA0501	C1073T	P358L	RNA polymerase beta-prime chain	DNA/RNA polymerase
JMUB217-12	6	*fruB*	SA0654	A482G	D161G	Fructose 1-phosphate kinase	Glycolysis
JMUB217-13	6	*fruB*	SA0654	C632A	A211E	Fructose 1-phosphate kinase	Glycolysis
JMUB217-14	8	*rpoB*	SA0500	A1729T	N577Y	RNA polymerase beta chain	DNA/RNA polymerase
JMUB217-15	12	*rpoC*	SA0501	C2215A	R739C	RNA polymerase beta-prime chain	DNA/RNA polymerase
JMUB217-16	12	*rpoC*	SA0501	CCA1856-7Ins	F619_N620insH	RNA polymerase beta-prime chain	DNA/RNA polymerase
JMUB217-17	16	*rpoC*	SA0501	G2317A	E773K	RNA polymerase beta-prime chain	DNA/RNA polymerase
JMUB217-18	24	*rpoC*	SA0501	T1463G, G2019T	V488G, K673N	RNA polymerase beta-prime chain	DNA/RNA polymerase
JMUB217-19	24	*rpoC*	SA0501	G2848C	G950R	RNA polymerase beta-prime chain	DNA/RNA polymerase
JMUB217-20	24	*rpoB*	SA0500	T3047A, C3126G	V1016E, H1042Q	RNA polymerase beta chain	DNA/RNA polymerase
JMUB217-21	24	*guaA*	SA0376	TATC744-7Del	I249fs	GMP synthase	Purine biosynthesis pathway
JMUB217-22	32	*rpoB*	SA0500	A1935C	Q645H	RNA polymerase beta chain	DNA/RNA polymerase
JMUB217-23	64	*rpoB*	SA0500	A2786C	H929P	RNA polymerase beta chain	DNA/RNA polymerase
JMUB217-24	256	*rpoC*	SA0501	G1493A	G498D	RNA polymerase beta-prime chain	DNA/RNA polymerase
JMUB1293-1	8	*HP3*	SA0224	G209T	T70K	Hypothetical protein, similar to 3-hydroxyacyl-CoA	Unknown function
		HP4	B5M25_04435	G234A	L78L	Phage tail protein	Unknown function
		intergenic-2	between SA1691 (*sgtB*) and SA1692	A48G	-	between monofunctional glycosyltransferase (mgt) and hypothetical protein	Intergenic region
JMUB1293-2	8	*folC*	SA1487	C574T	P192S	Folylpolyglutamate synthase	Folate biosynthesis pathway
JMUB1293-3	12	*vraT*	SA1702	G374A	T125I	Conserved hypothetical protein	Membrane protein associated with glycopeptide resistance
		HP4	B5M25_04435	G234A	L78L	Phage tail protein	Unknown function
JMUB1293-4	12	*vraT*	SA1702	G521T	P174Q	Conserved hypothetical protein	Membrane protein associated with glycopeptide resistance

Table 5-continued

Mutant strain	Oxacillin MIC (μg/mL)	Mutated gene	Locus tag in N315	Nucleotide change	Amino acid change	Product	Functional category
JMUB1293-5	16	*HP3*	SA0224	G209T	T70K	Hypothetical protein, similar to 3-hydroxyacyl-CoA	Unknown function
		HP4	B5M25_04435	G234A	L78L	Phage tail protein	Unknown function
		intergenic-2	between SA1691 (*sgtB*) and SA1692	A48G	-	Monofunctional glycosyltransferase/Uncharacterized protein SA1692	Intergenic region
JMUB1293-6	16	*HP3*	SA0224	G209T	T70K	Hypothetical protein, similar to 3-hydroxyacyl-CoA	Unknown function
		HP4	B5M25_04435	G234A	L78L	Phage tail protein	Unknown function
		intergenic-2	between SA1691 (*sgtB*) and SA1692	A48G	-	Monofunctional glycosyltransferase/Uncharacterized protein SA1692	Intergenic region
JMUB1293-7	24	*rpoC*	SA0501	C2783T	A928V	RNA polymerase beta-prime chain	DNA/RNA polymerase
JMUB1293-8	32	*HP5*	SA2220	A955G	Y319H	Conserved hypothetical protein	Unknown function
JMUB1293-9	256	*ftsH*	SA0469	G1627A	Q543*	Cell-division protein	Protein quality control
JMUB1283-1	1	*guaA*	SA0376	C889T	Q297*	GMP synthase	Purine biosynthesis pathway
		intergenic-3	between E8M03_00305 and E8M03_00310 (*hsdR*)	A6G	-	Hypothetical protein/type I restriction endonuclease subunit R	Intergenic region
JMUB1283-2	1.5	*rsh*	SA1460	C1255Del, C1254Ins	A419fs, Y418fs	GTP pyrophosphokinase	Purine biosynthesis pathway
		HP6	SA0204	T449A	E150V	FMN-dependent NADH-azoreductase	Unknown function
JMUB1283-3	3	*HP7*	SA2275	A450G	G150G	Uncharacterized lipoprotein SA2275	Unknown function
		intergenic-1	between SA0499 and SA500 (*rpoB*)	T173A	-	Conserved hypothetical protein/RNA polymerase beta chain	Intergenic region
JMUB1283-4	4	*rpoB*	SA0500	G3374A	P1125L	RNA polymerase beta chain	DNA/RNA polymerase
JMUB1283-5	6	*rpoB*	SA0500	T1874A	I625N	RNA polymerase beta chain	DNA/RNA polymerase
JMUB1283-6	12	*guaA*	SA0376	C680A	G227V	GMP synthase	Purine biosynthesis pathway
JMUB1283-7	24	*rpoB*	SA0500	C1930T	R644C	RNA polymerase beta chain	DNA/RNA polymerase
		intergenic-4	between SA2092 and SA2093 (*ssaA2*)	T159Ins	-	Hypothetical protein, similar to transcription regulator/Staphylococcal secretory antigen ssaA2	Intergenic region

Table 5-continued

Mutant strain	Oxacillin MIC (μg/mL)	Mutated gene	Locus tag in N315	Nucleotide change	Amino acid change	Product	Functional category
JMUB1283-8	128	*rpoB*	SA0500	G2780A	G927D	RNA polymerase beta chain	DNA/RNA polymerase
JMUB1284-1	256	*guaA*	SA0376	G944T	S315*	GMP synthase	Purine biosynthesis pathway
		HP8	SA0507	A1089T	K363N	Hypothetical protein, similar to N-acyl-L-amino acid amidohydrolase	Unknown function
JMUB1285-1	6	*ptsI*	SA0935	C539A	G180V	Phosphoenolpyruvate-protein phosphotransferase	Glycolysis
JMUB1289-1	4	*prs*	SA0458	A602C	D201A	Ribose-phosphate pyrophosphokinase	Pentose phosphate pathway
		intergenic-5	between SAS044 and SA1196	G114T	-	Probable tautomerase SA1195.1/Hypothetical protein, similar to DNA-damage repair protein	Intergenic region
JMUB1289-2	4	*HP9*	SA1389	G134T	A45E	Conserved hypothetical protein	Unknown function
JMUB1301-1	6	*guaA*	SA0376	C158T	G53D	GMP synthase	Purine biosynthesis pathway
JMUB1301-2	8	*pykA*	SA1520	G41A	S14L	Pyruvate kinase	Glycolysis
JMUB1301-3	8	*guaA*	SA0376	T1498A	Y500N	GMP synthase	Purine biosynthesis pathway
JMUB1301-4	16	*rpoC*	SA0501	T1482A	D494E	RNA polymerase beta-prime chain	DNA/RNA polymerase
JMUB1311-1	3	*vraT*	SA1702	G374A	T125I	Conserved hypothetical protein	Membrane protein associated with glycopeptide resistance
JMUB1972-1	3	*HP10*	SA2091	G651A	I217I	Hypothetical protein	Unknown function
JMUB1972-2	12	*rpoC*	SA0501	G3190A	V1064I	RNA polymerase beta-prime chain	DNA/RNA polymerase
JMUB1972-3	12	*rpoC*	SA0501	C1244T	A415V	RNA polymerase beta-prime chain	DNA/RNA polymerase
JMUB1972-4	24	*rpoC*	SA0501	A2295C,T2407A	L765F, Y803N	RNA polymerase beta-prime chain	DNA/RNA polymerase
JMUB1972-5	48	*rpoB*	SA0500	G2783A	R928Q	RNA polymerase beta chain	DNA/RNA polymerase
JMUB1976-1	6	*rpoC*	SA0501	G2351A	T784I	RNA polymerase beta-prime chain	DNA/RNA polymerase
JMUB1978-1	6	*clpX*	SA1498	A182C	L61*	ATP-dependent Clp protease ATP-binding subunit ClpX	Protein quality control
		HP11	E8M03_07940	T61C	I21V	AAA family ATPase	Unknown function

Table 5-continued

Mutant strain	Oxacillin MIC (μg/mL)	Mutated gene	Locus tag in N315	Nucleotide change	Amino acid change	Product	Functional category
JMUB1979-1	3	*HP12*	SA2481	G952A	A318T	conserved hypotehtical protein	Unknown function
		intergenic-6	between SA1269 (*norB*) and SA1267 (*ebhA*)	C37T	-	Quinolone resistance protein NorB/Extracellular matrix-binding protein EbhA	Intergenic region
JMUB1979-2	64	*clpX*	SA1498	T207Ins	H70fs	ATP-dependent Clp protease ATP-binding subunit ClpX	Protein quality control
		HP13	E8M03_07915	T178C	M60V	Hypothetical protein	Unknown function
		ebhA	SA1267	G14762T	G4921V	Extracellular matrix-binding protein EbhA	Extracellular matrix protein
		HP12	SA2481	G952A	A318T	Conserved hypotehtical protein	Unknown function
		intergenic-6	between SA1269 (*norB*) and SA1267 (*ebhA*)	C37T	-	Quinolone resistance protein NorB/Extracellular matrix-binding protein EbhA	Intergenic region
JMUB1280-1	48	*prs*	SA0458	C169T	G57S	Ribose-phosphate pyrophosphokinase	Pentose phosphate pathway
		HP14	SA0975	A420Ins	V141fs	Conserved hypothetical protein	Unknown function
		mraZ	SA1021	T133C	L45L	Transcriptional regulator MraZ	Transcriptional regulator
JMUB1281-1	3	*thrS*	SA1506	G1487A	P496L	Threonine--tRNA ligase	tRNA systhesis
JMUB1281-2	3	*rpoB*	SA0500	C1898T	S633L	RNA polymerase beta chain	DNA/RNA polymerase
		rpoC	SA0501	C2186T	P729L	RNA polymerase beta-prime chain	DNA/RNA polymerase
JMUB1281-3	4	*gltX*	SA0486	AGA333-5Del	E114L	Glutamate--tRNA ligase	tRNA systhesis
JMUB1281-4	8	*clpP*	SA0723	G5A	G2E	ATP-dependent Clp protease proteolytic subunit	Protein quality control
JMUB1281-5	16	*fruB*	SA0654	C811T	Q271*	Fructose 1-phosphate kinase	Glycolysis
		HP15	SA1815	T265G	T89P	Hypothetical protein, similar to Na+-transporting ATP synthase	Unknown function
		sgtB	SA1691	C556T	Q186*	Monofunctional glycosyltransferase	Peptidoglycan biosynthesis
JMUB1281-6	24	*rpoB*	SA0500	C1898T	S633L	RNA polymerase beta chain	DNA/RNA polymerase
JMUB1281-7	32	*guaA*	SA0376	G1158A	V386V	GMP synthase	Purine biosynthesis pathway
JMUB1281-8	256	*guaA*	SA0376	C973T	Q325*	GMP synthase	Purine biosynthesis pathway

Table 5-continued

Mutant strain	Oxacillin MIC (μg/mL)	Mutated gene	Locus tag in N315	Nucleotide change	Amino acid change	Product	Functional category
JMUB1286-1	4	*guaA*	SA0376	C806T	G269D	GMP synthase	Purine biosynthesis pathway
JMUB1286-2	24	*hprT*	SA0468	G308A	T103I	Hypoxanthine-guanine phosphoribosyltransferase	Purine biosynthesis pathway
JMUB1288-1	6	*clpP*	SA0723	ATAA184-7Del	L62fs	ATP-dependent Clp protease proteolytic subunit	Protein quality control
JMUB1292-1	6	*rpoC*	SA0501	A3191C	V1064G	RNA polymerase beta-prime chain	DNA/RNA polymerase
		dnaE	SA1525	T337A	F113I	DNA polymerase III subunit alpha	DNA/RNA polymerase
		intergenic-7	between SA0369 (*tnp*) and SA0531 (*proP*)	A121T	-	Transposase for IS1181/Putative proline/betaine transporter	Intergenic region
JMUB1292-2	6	*clpP*	SA0723	G101C	S34*	ATP-dependent Clp protease proteolytic subunit	Protein quality control
		HP16	SA2095	G20C	G7A	Hypothetical protein, similar to D-octopine dehydrogenase	Unknown function
JMUB1292-3	6	*clpP*	SA0723	G453T	N151K	ATP-dependent Clp protease proteolytic subunit	Protein quality control
		HP17	SA0330	A509G	I170T	Hypothetical protein, similar to ribosomal-protein-serine N-acetyltransferase	Unknown function
JMUB1292-4	6	*clpP*	SA0723	G453T	N151K	ATP-dependent Clp protease proteolytic subunit	Protein quality control
		HP17	SA0330	A509G	I170T	Hypothetical protein, similar to ribosomal-protein-serine N-acetyltransferase	Unknown function
JMUB1296-1	12	*prs*	SA0458	G951T	S317R	Ribose-phosphate pyrophosphokinase	Pentose phosphate pathway
JMUB1296-2	32	*rpoB*	SA0500	A1874T	I625N	RNA polymerase beta chain	DNA/RNA polymerase
		HP18	SA2318	C338T	A113V	Hypothetical protein, similar to L-serine dehydratase	Unknown function
JMUB1296-3	48	*rpoB*	SA0500	A1874T	I625N	RNA polymerase beta chain	DNA/RNA polymerase
		HP18	SA2318	C338T	A113V	Hypothetical protein, similar to L-serine dehydratase	Unknown function
JMUB1302-1	24	*rsh*	SA1460	C781T	Q261*	GTP pyrophosphokinase	Purine biosynthesis pathway

Table 5-continued

Mutant strain	Oxacillin MIC (μg/mL)	Mutated gene	Locus tag in N315	Nucleotide change	Amino acid change	Product	Functional category
JMUB1303-1	1.5	*rpoB*	SA0500	C812T	G271D	RNA polymerase beta chain	DNA/RNA polymerase
		intergenic-8	between SA1447and SA1446 (*alaS*)	G151T	-	between hypothetical protein, similar to deoxyribonuclease and hypothetical protein, similar to alanine--tRNA ligase	Intergenic region
JMUB1303-2	8	*prs*	SA0458	T583G	T195P	Ribose-phosphate pyrophosphokinase	Pentose phosphate pathway
JMUB1310-1	3	*hprT*	SA0468	G3A	M1I	Hypoxanthine-guanine phosphoribosyltransferase	Purine biosynthesis pathway
JMUB1310-2	6	*rpoC*	SA0501	G2821A	R941C	RNA polymerase beta-prime chain	DNA/RNA polymerase
JMUB1310-3	8	*clpP*	SA0723	C341A	A114E	ATP-dependent Clp protease proteolytic subunit	Protein quality control
JMUB1310-4	8	*rpoC*	SA0501	C1480T	D494N	RNA polymerase beta-prime chain	DNA/RNA polymerase
JMUB1310-5	12	*rsh*	SA1460	982T	R328*	GTP pyrophosphokinase	Purine biosynthesis pathway
JMUB1310-6	16	*tilS*	SA0467	T1287G	G429G	tRNA(Ile)-lysidine synthase	tRNA systhesis
JMUB1310-7	24	*fbaA*	SA1927	C22A	E8*	Fructose-bisphosphate aldolase	Glycolysis
JMUB1310-8	24	*yjbH*	SA0860	T740Ins	I247fs	UPF0413 protein SA0860	Protein quality control
JMUB1310-9	256	*rpoB*	SA0500	C2780T	G927D	RNA polymerase beta chain	DNA/RNA polymerase
JMUB1312-1	24	*rpoC*	SA0501	C1004T	R335H	RNA polymerase beta-prime chain	DNA/RNA polymerase
JMUB1314-1	4	*clpX*	SA1498	C977T	A326V	ATP-dependent Clp protease ATP-binding subunit ClpX	Protein quality control
JMUB1314-2	48	*lysS*	SA0475	A1403C	I468S	Lysine--tRNA ligase	tRNA systhesis
JMUB1316-1	12	*prs*	SA0458	T674C	I225T	Ribose-phosphate pyrophosphokinase	Pentose phosphate pathway
JMUB1316-2	48	*rpoB*	SA0500	C2908A	Q970K	RNA polymerase beta chain	DNA/RNA polymerase

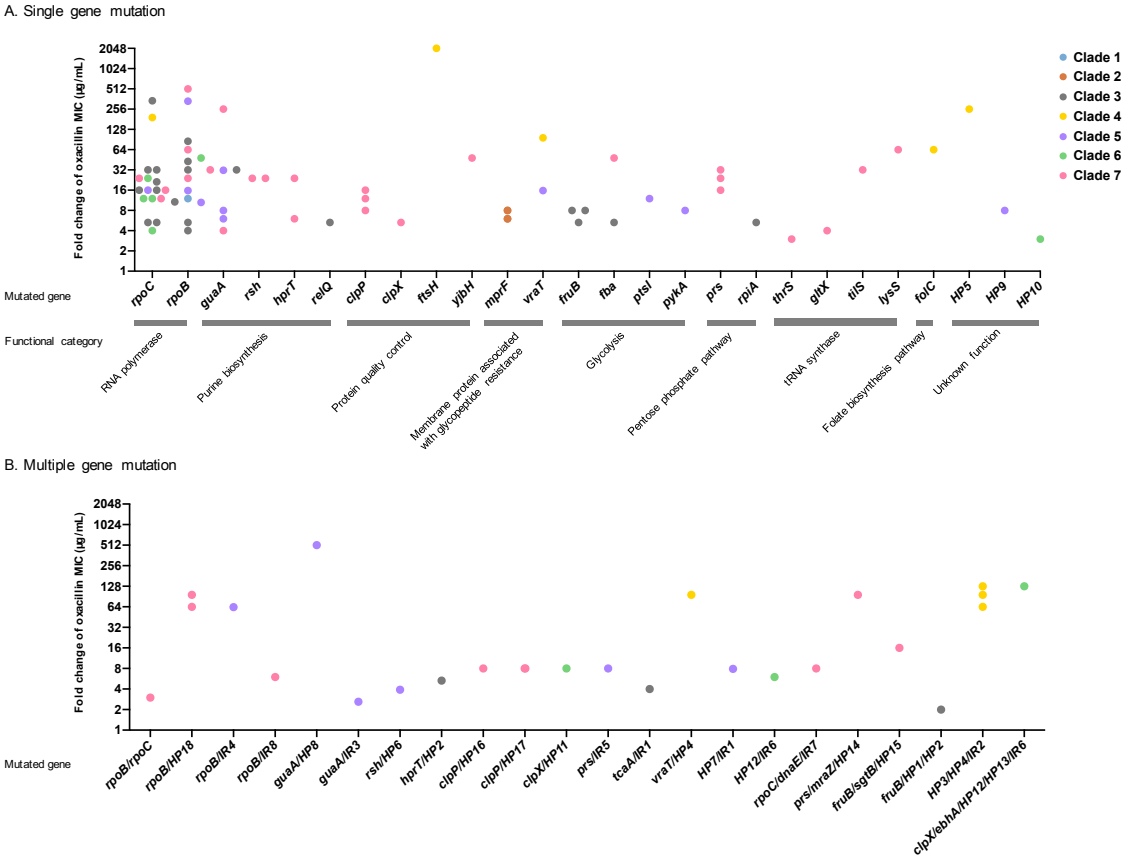

Fig 2: Relationship between mutated genes and fold changes of oxacillin minimum inhibitory concentrations (MICs) in mutants with reduced oxacillin susceptibility. Each mutant with reduced oxacillin susceptibility is represented by a filled, color-coded circle indicating different phylogenetic clades. (A) Fold changes of oxacillin MICs in mutants carrying single or double mutations in same gene. (B) Fold changes of oxacillin MICs in mutants carrying single or double mutations in different genes.

4-4. Increased *mecA* expression was responsible for reduced oxacillin susceptibility in OS-MRSA

To understand the role of the identified mutations in reduced oxacillin susceptibility, the JMUB217 strain (ST772-SCCmec type V) and its mutants were selected as representative strains for further study because we could generate 24 mutants carrying 26 variants in 11 genes and an intergenic region could be derived from the parental strain. In addition, the oxacillin MICs for these 24 mutants ranged 1.5–256 μg/mL, versus 0.75 μg/mL for JMUB217, and the JMUB217 strain appeared to be the only strain harboring two types of *bla* operons in its genome. First, we created a *mecA*-overexpressing mutant to investigate whether changes in its expression affect oxacillin susceptibility in OS-MRSA. pKAT containing *mecA* and its native promoter was introduced into JMUB217 to generate the *mecA*-overexpressing mutant JMUB217 (pmecA), and the generated mutant exhibited an increase in its oxacillin MIC from 0.75 to 48 μg/mL (Table 6). Next, we created *mecA*-knockout mutants of JMUB217 and measured the oxacillin MIC. The oxacillin MIC slightly decreased from 0.75 to 0.38 μg/mL following *mecA* deletion (Table 6), indicating that the presence of *mecA* itself confers a low level of oxacillin tolerance in JMUB217. We also overexpressed *mecA* in the *mecA*-deleted mutant JMUB217 (Δ*mecA*), resulting in an increment of the oxacillin MIC to 48 μg/mL, similar to that of the *mecA*-overexpressing mutant JMUB217 (pmecA).

Moreover, we also generated *mecA*-knockout strains from three oxacillin-resistant mutants (JMUB217-11, JMUB217-22, and JMUB217-24) carrying $RpoC^{P358L}$, $RpoB^{G645H}$, and $RpoC^{G498D}$, respectively, and found that their oxacillin MICs decreased to 0.38 μg/mL from 4, 32, and 256 μg/mL, respectively, similar to that of the *mecA*-knockout mutants of JMUB217 (Table 6). These results indicated that *mecA* expression is a key factor promoting reduced oxacillin susceptibility in OS-MRSA.

Table 6: Oxacillin MIC of *mecA* overexpression and deletion derivatives in OS-MRSA JMUB217 and its oxacillin-reduced susceptibility mutants

Strain	Description	Oxacillin MIC (μg/mL)
JMUB217	parent	0.75
	p*mecA*	48
	ΔmecA	0.38
	ΔmecA -p*mecA*	48
JMUB217-11	$RpoC^{P358L}$	4
	$RpoC^{P358L}$ -Δ*mecA*	0.38
JMUB217-22	$RpoB^{Q645H}$	32
	$RpoB^{Q645H}$ -*ΔmecA*	0.38
JMUB217-24	$RpoC^{G498D}$	256
	$RpoC^{G498D}$ -*ΔmecA*	0.38

4-5. Correlation between *mecA* expression levels and oxacillin MICs in mutants with reduced oxacillin susceptibility

To examine the correlation between *mecA* expression and oxacillin susceptibility, *mecA* expression was analyzed in 23 JMUB217-derived mutants with reduced susceptibility to oxacillin in the presence and absence of oxacillin (0.1 μg/mL). In this analysis, one mutant carrying $GuaA^{I249fs}$ (JMUB217-21) was omitted because of its extremely slow-growing phenotype. The results indicated that the natural expression of *mecA* in all mutants was extremely low in the absence of oxacillin, whereas its expression in the presence of oxacillin was significantly increased in the mutant and wild-type strains in line with the level of oxacillin resistance. The correlation coefficient between *mecA* expression and the oxacillin MIC was 0.78 ($p < 0.0001$) (Fig 3). Although the correlation coefficient was relatively high, the resistant mutant with the highest oxacillin MIC did not display the strongest *mecA* expression. Specifically, JMUB217-24 had the highest oxacillin MIC of 256 μg/mL, but it had lower *mecA* levels than JMUB217-22, JMUB217-19, JMUB217-20, and JMUB217-17, which had oxacillin MICs of 32, 24, 24, and 16 μg/mL, respectively (Table 5, Fig 3), indicating that *mecA* expression was not the only cause of oxacillin resistance in the mutants.

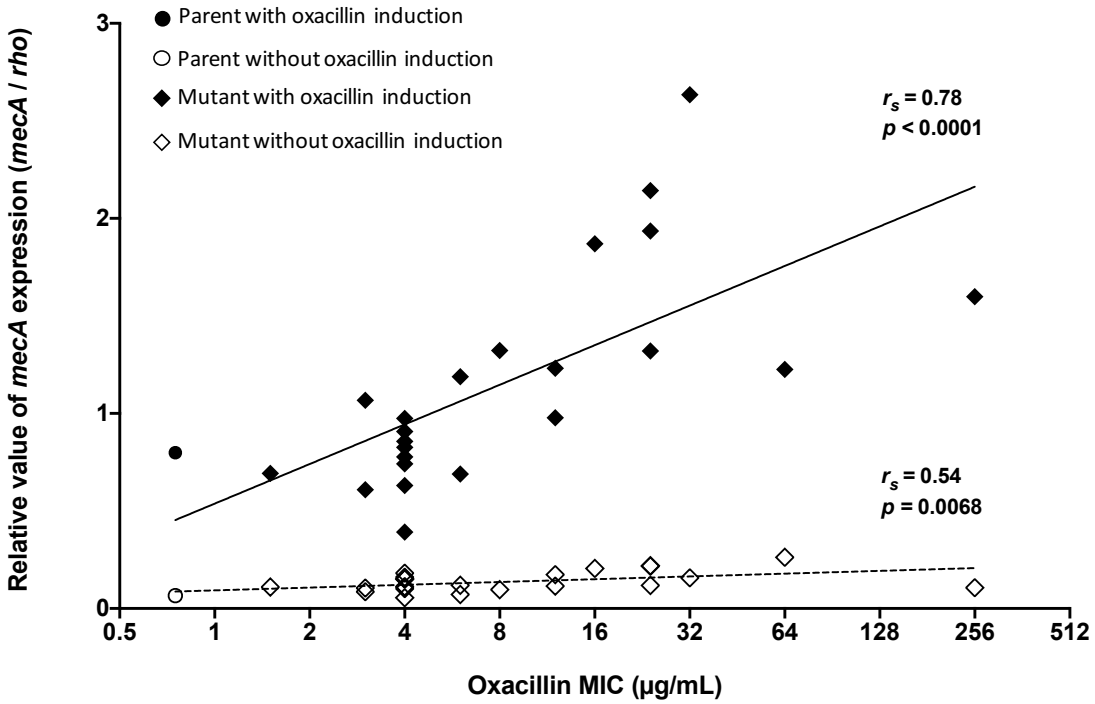

Fig 3: Relationship between oxacillin minimum inhibitory concentrations (MICs) and *mecA* expression levels in the oxacillin-susceptible methicillin-resistant *S. aureus* (OS-MRSA) strain JMUB217 and its mutants with reduced oxacillin susceptibility.

Expression of *mecA* in the OS-MRSA strain JMUB217 (circle) and its oxacillin-reduced susceptibility mutants (diamond) with (black color) and without oxacillin induction (white color) were measured using qRT-PCR and shown as fold changes normalized to *rho* expression. The correlation between oxacillin MICs and *mecA* expression was evaluated using Spearman's correlation coefficient (r_s) analysis. A two-sided p-value < 0.05 was considered significant.

4-6. The *bla* operon is involved in the reduced susceptibility to oxacillin

A previous study suggested that *blaI* expression levels were associated with reduced oxacillin susceptibility in OS-MRSA isolates (30). To understand the mechanism by which the *bla* operons affect oxacillin susceptibility in the tested OS-MRSA strains, we first generated *blaI*-knockout mutants. Our whole-genome sequencing analysis

revealed that *bla* regulatory genes (*blaI* and *blaR1*) were carried by 36 of the 43 (84%) OS-MRSA isolates, and the *bla* operons could be classified into two genotypes, both of which were carried by JMUB217 (Table 4). Therefore, we deleted one or both of these operons in JMUB217 to generate single and double *blaI*-knockout mutants, respectively, and determined their penicillin G and oxacillin MICs. Knockout of *blaI*-1 or *blaI*-2 alone did not significantly affect the penicillin G and oxacillin MICs of the mutants, whereas mutants in both genes were deleted exhibited an increase in the penicillin G MIC from 1.5 to 8 μg/mL (Table 7). Although that of oxacillin was also increased from 0.75 to 2 μg/mL, the double-gene-knockout mutants remained susceptible to oxacillin according to the CLSI criteria. These results indicated that the influence of *blaI* on oxacillin susceptibility is limited, and other genetic factors may more strongly affect oxacillin susceptibility than *blaI* in OS-MRSA.

Table 7: MIC of *blaI* deletion derivatives in OS-MRSA JMUB217

	MIC (μg/mL)	
Strain	Penicillin G	Oxacillin
JMUB217	1.5	0.75
JMUB217Δ*blaI*-1	1.5	1.5
JMUB217Δ*blaI*-2	2	1
JMUB217Δ*blaI*-1/-2	8	2

4-7. Transcriptome analysis revealed stringent response-like gene regulation in the oxacillin resistant mutants

To understand the mechanism by which mutations in the oxacillin-resistant mutants affect transcriptional profiles and ultimately bias gene regulation toward the expression of oxacillin resistance in the OS-MRSA mutants, the whole-genome expression profiles of five representative mutants of JMUB217 (JMUB217-7 carrying $RpiA^{A64E}$ [oxacillin MIC = 4 μg/mL], JMUB217-11 carrying $RpoC^{P358L}$ [oxacillin MIC = 4 μg/mL], JMUB217-18 carrying $RpoC^{V488G,K673N}$ [oxacillin MIC = 24 μg/mL], JMUB217-20 carrying $RpoB^{V1016E,H1042Q}$ [oxacillin MIC = 24 μg/mL], and JMUB217-22 carrying $RpoB^{Q645H}$ [oxacillin MIC = 32 μg/mL]) were analyzed and compared with that of the parental strain JMUB217. These transcriptome analyses were performed under both oxacillin-induced and drug-free growth conditions. As shown in Fig 4, in concordance with the qRT-PCR data, *mecA* expression was significantly increased in the mutants grown under oxacillin induction (Fig 4A). BlaR1, an activator of *blaZ* and *mecA* transcription that regulates the increased expression of the resistance genes in the mutants, was also highly expressed in the mutants (Fig 4A). In addition to *mecA* gene, other antibiotic resistance genes, including *bla* operons, the bacitracin resistance gene *bcrAB*, and the aminoglycoside resistance gene *aac*(6′)-*aph*(2″), were also upregulated not only

in the mutants but also in the parent strain JMUB217 under oxacillin induction. Only the expression of *dfrG* gene was increased specifically in the mutants.

In addition to the aforementioned alterations potentially associated with oxacillin resistance in the generated mutants, classic stringent response-like gene regulation was observed via transcriptomic analysis. As shown in Fig 4, significant downregulation of genes associated with purine biosynthesis, glycolysis, the pentose phosphate pathway, folate biosynthesis, protein quality control, tRNA synthesis, and peptidoglycan synthesis was found in a series of resistance mutants carrying mutations in *rpoC*, *rpoB*, and *rpiA*. The downregulation of genes involved in glycolysis, the pentose phosphate pathway, and folate and purine biosynthesis was clearly linked with lower intracellular GTP levels (Fig 4G–J). Meanwhile, *rsh* gene was downregulated in our studied strains (Fig 4I). The downregulation of *rsh* may resulted in the decreased expression of (p)ppGpp-mediated stringent response of *S. aureus*. The downregulation of *rsh* resembled the partial loss of function caused by mutation of *rsh*, as demonstrated by our whole-genome sequencing analysis (frameshift mutation [$Rsh^{Y418fs,A419fs}$] or nonsense mutation [Rsh^{Q261*} and Rsh^{R328*}]).

Some additional classic stringent-like responses gene expression profiles were found. The mutants with reduced oxacillin susceptibility also exhibited downregulation of genes involved in protein quality control and tRNA synthesis. Notably, *clpP*, *clpX*, and

ftsH (mutations of which were identified in the mutants with reduced oxacillin susceptibility) were significantly downregulated in mutants with reduced oxacillin susceptibility (Fig 4B). *S. aureus* carries at least 25 tRNA synthesis genes, and our results indicated that four of these genes were mutated in the mutants with reduced oxacillin susceptibility. In addition to the downregulation of genes involved in GTP metabolism and protein quality control, 16 of 25 tRNA genes were also downregulated in at least one of the five studied mutants grown with or without oxacillin induction (Fig 4C). These changes in gene expression might contribute to oxacillin resistance.

Transcriptome profiles also suggested that mutations in *rpoBC* and *rpiA* affect the structure of peptidoglycan, as implicated by the upregulation of *mecA* and *sgtB* and downregulation of *murBJY*, *femABX*, *pbp4*, *ftsW*, and *rodA* in at least one of the mutants grown with or without oxacillin induction (Fig 4K). Furthermore, changes in the expression of genes involved in autolysis were observed in strains carrying mutated *rpoBC* and *rpiA*; specifically, *lytM* and *sle1* were upregulated, whereas *lytH*, *isaA*, and *ssaA* were downregulated (Fig 4D). Differential changes of peptidoglycan synthesis and autolysis may alter the structure of peptidoglycan and subsequently cause oxacillin resistance.

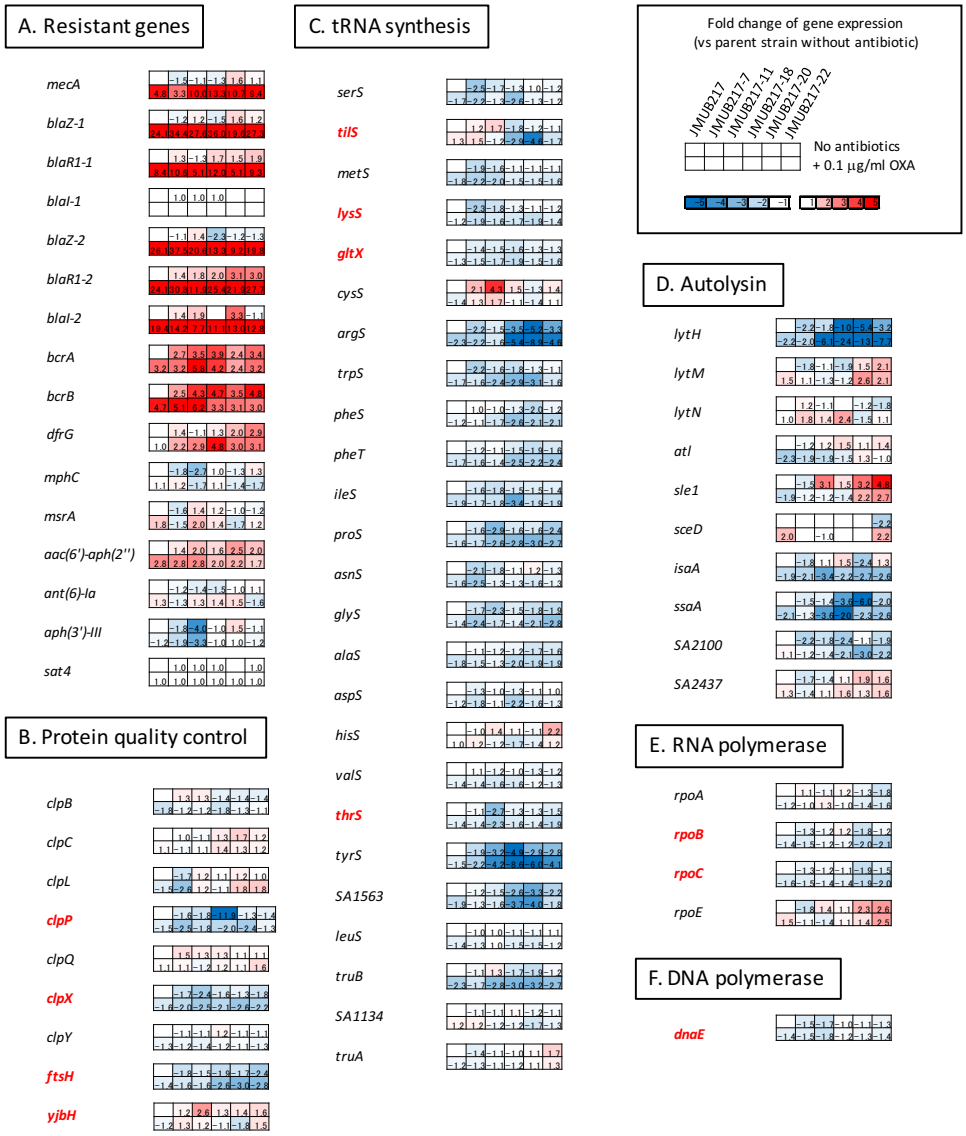

Fig 4: Gene expression profiles of the representative oxacillin-susceptible methicillin-resistant *S. aureus* (OS-MRSA) strain JMUB217 and its mutants in the presence/absence of oxacillin (0.1 μg/mL).

Differentially expressed genes between the parental strain JMUB217 and its mutants were classified according to functional categories, including (A) antibiotic resistance, (B) protein quality control, (C) tRNA synthesis, (D) autolysis, (E) RNA polymerase activity, (F) DNA polymerase activity, (G) glycolysis, (H) the pentose phosphate pathway, (I) purine biosynthesis, (J) folate biosynthesis, and (K) peptidoglycan biosynthesis. The color scales indicate log2-fold changes of transcriptional expression compared with that in the parental strains JMUB217 without induction. The white panel denotes the growth conditions for each strain. Genes identified in the *in vitro* mutation analysis of the mutants with reduced oxacillin susceptibility are shown in red.

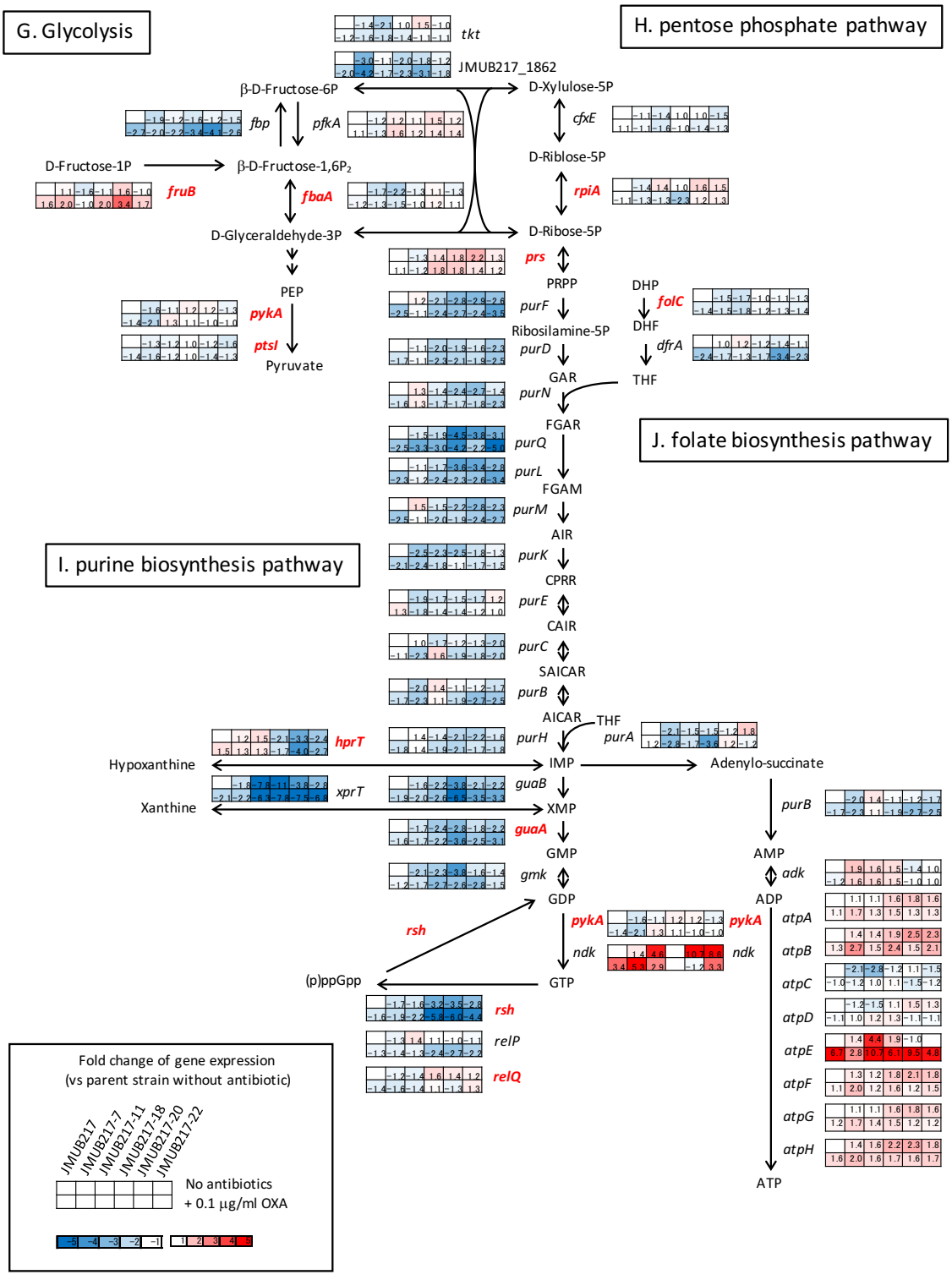

Fig 4-continued.

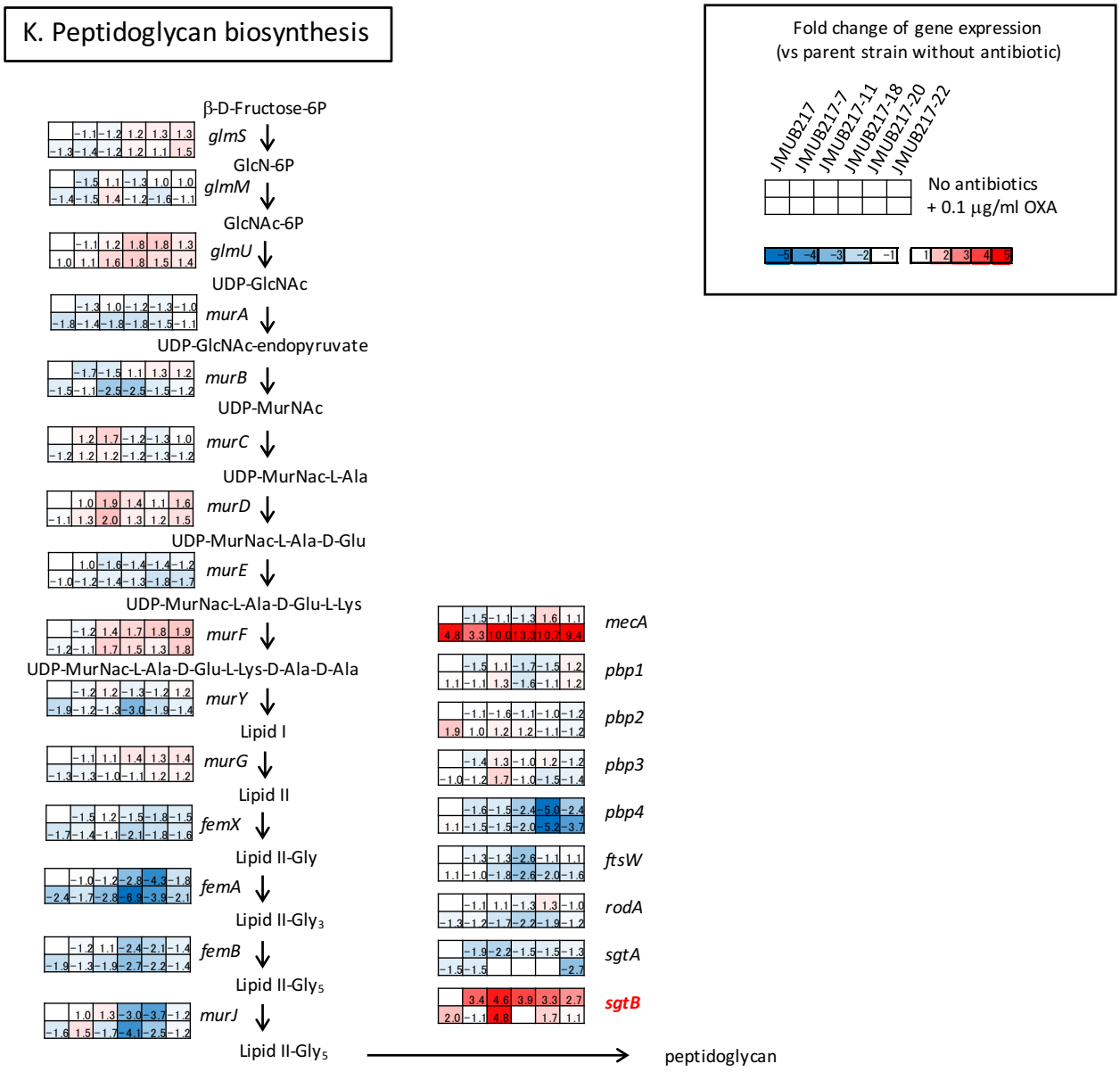

Fig 4-continued.

4-8. The mutants with reduced oxacillin susceptibility did not display a slow-growth phenotype

Mutations in genes involved in the stringent stress response were found to be associated with slower growth rates (31). In addition, some β-lactam–resistant mutants generated *in vitro* were also reported to have slow growth rates or a phenotype of persistent infection (31-34). To investigate whether the mutations identified in the oxacillin-resistant mutants affect cell growth, the doubling time of 23 JMUB217-derived mutants was measured. Unexpectedly, excluding JMUB217-21 ($GuaA^{I249fs}$), all mutants had similar doubling times as the parental strain JMUB217 (Fig 5A).

4-9. Intercellular ATP accumulation in the resistant mutants

Some reports examining intercellular ATP levels in *S. aureus* in relation to stress responses found that lower cellular ATP production was associated with bacterial tolerance to several environmental stresses such as salt, cold, and antibiotics, and it could also induce the conversion of bacterial cells into persistent forms, including small colony variants (35, 36). Our transcriptomics study with the resistant mutants illustrated that several genes involved in purine biosynthesis and folate biosynthesis were clearly downregulated (Fig 4I and 4J), which was similar to the findings of Cassels *et al* (37). However, most all genes involved in ATP biosynthesis were significantly upregulated

(Fig 4J), and in agreement with this finding, intercellular ATP levels were significantly higher in the 23 JMUB217-derived mutants than in the parental strain JMUB217. In addition, a significant positive correlation was noted between intracellular ATP levels and oxacillin MICs ($r_s = 0.60$, $p = 0.0017$; Fig 5B). The upregulation of genes associated with ATP biosynthesis might represent one feedback response for complementing or restoring the normal cellular physiology.

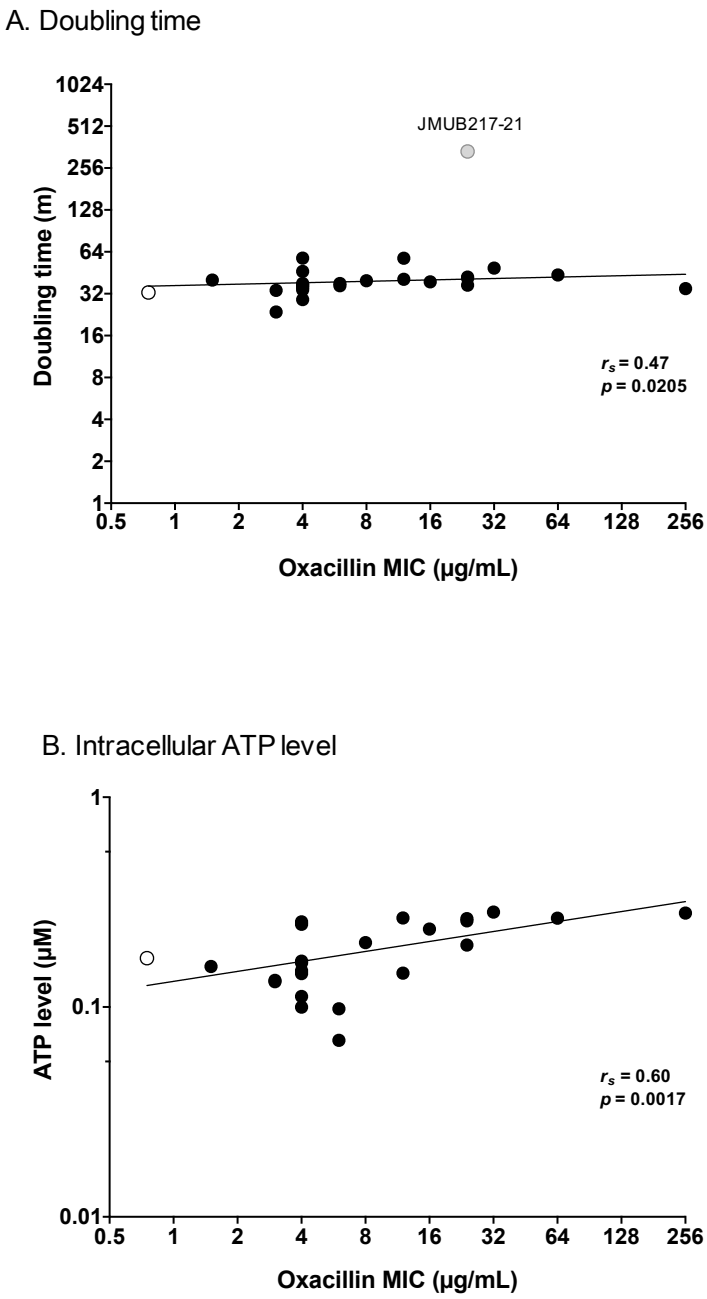

Fig 5: Correlations of the doubling time (A) and intracellular ATP level (B) with oxacillin minimum inhibitory concentrations (MICs) among the representative strain JMUB217 and its mutants.

Each dot represents an isolate. The open and black circles indicate the parent JMUB217 and JMUB217-derived mutants, respectively. The grey circle represents JMUB217-21 ($GuaA^{I249fs}$) exhibiting a slow-growth phenotype. The correlation between these variables was evaluated using Spearman's correlation coefficient (r_s) test. A two-sided p-value < 0.05 was deemed significant.

5. **Discussion**

Since its first description in 1991 (15, 38), OS-MRSA, which is related to borderline methicillin-resistant MRSA (39), has been frequently isolated in hospital and community settings with a prevalence rate of 0.62–33.7% (12, 15, 18, 40-42). The presence of OS-MRSA is currently a challenge in the clinical management of staphylococcal infections and requires great attention because is prone to be misidentified as MSSA via routine β-lactam susceptibility testing (10, 12, 14). Indeed, the majority of the OS-MRSA isolates used in this study were initially identified as MSSA according to the oxacillin susceptibility profile provided by the original laboratory despite carrying *mecA*. Similarly, susceptibility testing using cefoxitin, a stronger inducer of the *mecA* regulatory system than oxacillin that is used to detect methicillin resistance (43), failed to accurately identify most of the strains as *mecA*-positive OS-MRSA (Table 1). These observations suggest that a combination of oxacillin and cefoxitin susceptibility tests, as previously recommended (44), or better yet detection of *mecA*, will be more reliable for the identification of MRSA.

Despite being phenotypically susceptible to oxacillin, β-lactam resistance can easily be induced in OS-MRSA (42, 45, 46). The mechanisms regulating oxacillin susceptibility in *S. aureus* appear to differ depending on the types of mutations and genetic basis of the individual isolates. Chen *et al*. reported that mutations in the MecI

binding site of the *mecA* promoter downregulated the expression of PBP2a and increased the susceptibility of ST59-SCC*mec* type V strains to oxacillin (47). Meanwhile, they demonstrated that mutation of the ribosome-binding site of *mecA* in an ST59-SCC*mec* type IV strain attenuated its oxacillin resistance. Nevertheless, these mutations affect only the oxacillin resistance of strains from the ST59 background, whereas mutations in the same locus barely affected the β-lactam resistance levels of isolates with different genetic backgrounds, such as COL (ST250) and CH482 (ST45) (48). Conversely, mutations in the *mecA* coding region conferred oxacillin resistance to OS-MRSA strains isolated in the US (46). These studies suggested that the inclusion of OS-MRSA strains with a wide range of genetic backgrounds is crucial for providing a comprehensive overview of the mechanism of oxacillin resistance of OS-MRSA.

Among the 118 variants identified in this study, most were previously reported to be involved in the heterogeneous-to-homogeneous alteration of β-lactam resistance in typical MRSA, including genes associated with RNA polymerase (RNAP; *rpoB* (33, 49) and *rpoC* (33, 50)), purine biosynthesis (*guaA* (33), *prs* (32, 33), *hprT* (32, 33), and *rsh* (32, 33, 51)), glycolysis (*pykA* (33), *fbaA* (33), and *fruB* (32)) protein quality control (*ftsH* (33), *clpX* (52, 53), and *clpP* (52, 53)), and tRNA synthase (*lysS* (33), and *gltX* (33)). In addition, synonymous mutations (*guaA*^{V386V}, *tilS*^{G429G}, HP7^{G150G}, and HP10^{I217I}) were identified in four oxacillin-reduced susceptibility mutants. The mutated genes might be

responsible for the increased oxacillin MICs of the mutants given that no other mutations were found in the coding regions of each strain even though these mutations resulted in no amino acid substitution. This finding was supported by the previous result that linking silent mutations in *rpoB* and *gyrA* genes with ciprofloxacin resistance in MRSA (54). Among the identified mutations, RNAP genes (*rpoBC*) were frequently mutated in oxacillin-resistant mutants from six of the seven phylogenetic clades, suggesting that *rpoB* and *rpoC* were the fundamental determinants of oxacillin resistance in OS-MRSA. Mutations in *rpoBC* resulted in increased cellular levels of the *mecA* gene product PBP2a (33), and this led us to further investigate the role of *mecA* in the oxacillin resistance phenotype of OS-MRSA.

Based on the current study results, the oxacillin susceptibility of OS-MRSA was postulated to be regulated by the expression levels of *mecA*. *mecA* transcription is strongly repressed by the *mecA* regulatory element MecI and *blaZ* repressor BlaI (55). Among the OS-MRSA isolates included in our study, intact *mecI* was detected in only one isolate (JMUB1293 carrying SCC*mec* type II), whereas *blaI* was harbored by 36 OS-MRSA strains, suggesting that *blaI* may be the key regulator of oxacillin susceptibility in our studied strains. In support of this result, previous reports also suggested that *blaI* was mainly responsible for regulating the oxacillin resistance phenotype in staphylococcal isolates including OS-MRSA (30, 56). However, single and double deletion of *blaI* in the

representative OS-MRSA strain JMUB217 resulted in only slight increases of the oxacillin MIC. This supported the presence of other undetermined regulatory factors that affect *mecA* expression.

Although oxacillin resistance in OS-MRSA was caused by increased *mecA* expression, the exact mechanism triggering *mecA* overexpression is unknown. In addition, *mecA* levels were not uniformly increased in JUMB217-derived oxacillin-resistant mutants compared with that in their corresponding susceptible parental strain, as determined by qRT-PCR. Therefore, we postulated that oxacillin resistance in OS-MRSA strains involves a more complex regulatory pathway than simply direct *mecA* signaling. The study results indicated that the fundamental mechanism of oxacillin resistance is closely related to the defined mutations. However, the expression profiles of stringent response elements in oxacillin-resistant mutants derived from this study were not indicative of the classic stringent response elicited by mupirocin treatment (57).

The stringent stress response governed by the alarmone (p)ppGpp is involved in the β-lactam resistance of MRSA (34, 51). Despite the (p)ppGpp-mediated classic stringent response, we demonstrated in our study that oxacillin resistance in OS-MRSA can also be induced by another pathway, which we coined "stringent response-like expression." This pathway is not associated with the accumulation of (p)ppGpp. Fig 6 depicts the proposed metabolic pathway of oxacillin resistance in OS-MRSA mutants.

Both our whole-genome comparative analysis and RNA-seq analysis suggested that the intracellular accumulation of (p)ppGpp may not be necessary for the induction of oxacillin resistance, but the lower intracellular GTP level may play a key role in oxacillin resistance, as evidenced by the downregulation of genes responsible for GTP biosynthesis (purine and folate biosynthesis, pentose phosphate biosynthesis, and glycolysis). During the classic stringent response, the cellular stresses resulting from amino acid starvation and mupirocin exposure induce the accumulation of uncharged (deacylated) tRNA (58). The uncharged tRNA in turn binds to the A (aminoacyl-tRNA) site of the 70S ribosome and activates Rsh to produce (p)ppGpp (59). In *E. coli*, the classic stringent response is elicited by the direct binding of (p)ppGpp to RNAP (60-62). However, in Firmicutes, including *B. subtilis* and *S. aureus*, (p)ppGpp does not directly regulate RNAP. Rather, (p)ppGpp synthesis reduces intracellular GTP levels, subsequently leading to the induction of the stringent response (63). Hence, mutations in genes involved in glycolysis, pentose phosphate biosynthesis, folate synthesis, and purine biosynthesis might mimic the (p)ppGpp-mediated reduction in intracellular GTP levels and induce "stringent response-like expression" (Fig 6). The non-accumulation of (p)ppGpp in this stringent response-like expression may be partly attributable to the stable/downregulated expression of the (p)ppGpp synthase genes *rsh* and *relPQ*, which were resulted from loss-of-function mutations in *rpoBC* or *rpiA*. The activation of stringent response-like

expression will subsequently increase the expression of *mecA* via *blaR1* upregulation, as well as influence the expression of genes involved in autolysis and peptidoglycan synthesis, all of which might result in oxacillin tolerance (Fig 6). In this study, we demonstrated that oxacillin-selected resistant mutants acquired a resistance mechanism distinct from that associated with mupirocin treatment, leading to strong β-lactam resistance.

Previous studies reported that the CodY regulon including blanched-chain amino acid (BCAA) biosynthesis operon *ilvDBC-leuABC-ilvA* is upregulated in the classic stringent response elicited by the amino-acid starvation (64). However, our transcriptional analysis revealed that the CodY regulon was not upregulated in the stringent response-like expression observed in the oxacillin resistant mutants. The non-induction of CodY regulon in the oxacillin-resistant mutants may be due to the intracellular BCAA level (Fig 6). Muller-Hinton media used in this study contains enough amount of BCAA to remain activating the negative regulator CodY in the resistant mutants. Indeed, the CodY regulon is strictly repressed in both Gram-negative and Gram-positive bacteria including *S. aureus* when intracellular levels of BCAA are sufficient to support growth (65-68). In addition, the gene expression profiles identified in the mutants with reduced oxacillin susceptibility were closely related to that of the (p)ppGpp

synthesis-deficient mutant that did not exhibit upregulation of the CodY regulon under both amino acid starvation and mupirocin treatment due to the RSH/RelA^{SA} mutation (69). RSH is a bifunctional enzyme possessing (p)ppGpp hydrolase and synthase activities, with the N-terminal (p)ppGpp hydrolase activity being essential for *S. aureus*. In this study, all four *rsh* mutations resulted in a premature stop codon or truncation (Q261*, R328*, A419fs/Y418fs), indicating the possible deficiency of (p)ppGpp synthase but normal N-terminal hydrolase activity. Moreover, our RNA-seq data also indicated that *rsh* was strictly downregulated in *rpoBC* and *rpiA* mutants, suggesting that the *mecA* overexpression-promoted oxacillin resistance in OS-MRSA is associated with a (p)ppGpp-deficient stress response.

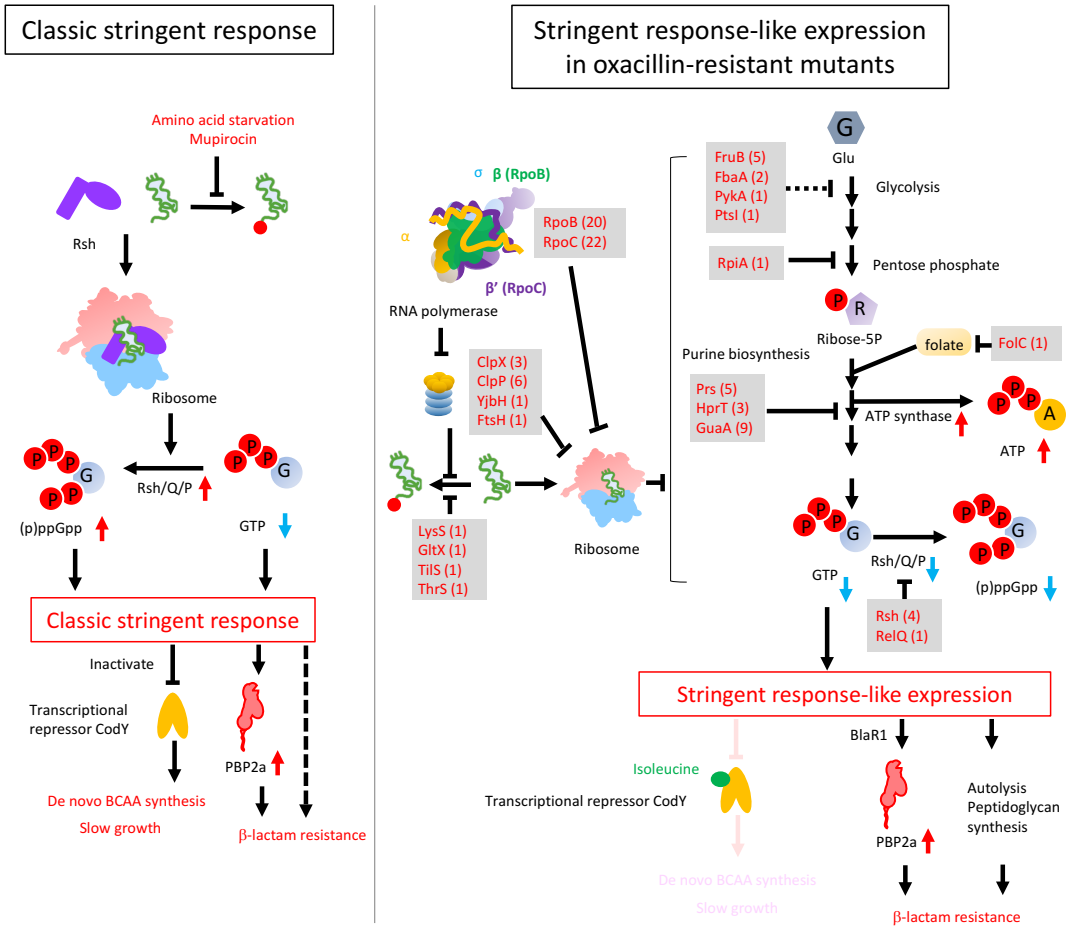

Fig 6: Proposed model of the mechanism of acquired resistance to oxacillin in oxacillin-susceptible methicillin-resistant *S. aureus* (OS-MRSA) mutants with reduced oxacillin susceptibility.

The red letter in the gray box represents a protein encoded by a gene found to be mutated in mutants with reduced oxacillin susceptibility, whereas the number of identified mutations is indicated within brackets. The mutations in certain genes affect multiple cellular pathways, resulting in lower expression of genes associated with GTP homeostasis without the accumulation of (p)ppGpp. Consequently, the decrease of GTP levels in the dNTP pool alters the expression of genes involved in antimicrobial resistance, autolysis and peptidoglycan synthesis. However, the stringent response-like expression does not induce CodY regulon because of the sufficient intracellular BCAA level. Conversely, the classic stringent response elicited by mupirocin treatment or amino acid starvation induces (p)ppGpp accumulation and decreases GTP levels in the dNTP pool. The fundamental mechanism of β-lactam resistance elicited by the mutation-derived stringent-like response is suggested to be closely related to that of the (p)ppGpp-mediated classic stringent response.

OS-MRSA is considered problematic in the clinical setting because the strain is prone to develop high-level β-lactam resistance during the course of antibiotic treatment (42, 45). Because the targets of antibiotics are generally essential proteins in bacteria, the acquisition of antibiotic resistance is usually associated with a fitness cost (70). In *S. aureus*, slow-growth phenotypes, including the formation of small colony variants, are associated with tolerance to antibiotics (71-74). Contrarily, most of the mutations identified in the JMUB217 strain (ST772-SCC*mec* type V) altered its oxacillin susceptibility without affecting its doubling time. This suggested that the mutations conferring reduced oxacillin susceptibility in OS-MRSA may incur only small fitness costs because of the complementary upregulation of ATP synthase genes. The increased expression of ATP biosynthesis genes was supported by the positive correlation between oxacillin MICs and intracellular ATP levels, which might explain the easy acquisition of oxacillin resistance in OS-MRSA. Nonetheless, chromosomal mutations in *rpoBC* and other genes involved in purine biosynthesis were identified in slow VISA strains (75, 76), indicating that the fitness cost of mutations may depend on the genetic background of individual strains.

This study aimed to determine the genetic pathways associated with the acquisition of oxacillin resistance in OS-MRSA isolates from diverse genetic backgrounds. Our results suggested that OS-MRSA was rendered oxacillin-resistant by

stringent response-like expression (a stress response similar to the classic stringent response that does not involve (p)ppGpp accumulation) and the ensuing expression of antibiotic resistance genes (e.g., *mecA*, *bla* operon). The relatively low fitness cost of the mutations may fuel the easy selection of oxacillin-resistant OS-MRSA mutants during the course of antimicrobial treatment. Therefore, *mecA* detection is important for differentiating OS-MRSA from MSSA in clinical microbiology laboratories to prevent misdiagnosis. Simple and low-cost systems for *mecA* detection could represent promising modalities for the improved treatment of MRSA infection.

6. Conclusion

This study set out to determine the genetic pathway in acquisition of oxacillin resistance in OS-MRSA with diverse genetic backgrounds. Our results suggested that OS-MRSA was rendered oxacillin-resistant by the stringent response-like expression (a stress response similar to the classic stringent response but without involving (p)ppGpp accumulation) and the ensuing expression of antibiotic resistance genes (*mecA*, *bla* operon, etc.). The relatively low fitness cost of the mutations may fuel the easy selection of resistant mutants of OS-MRSA during the course of antimicrobial treatment. Therefore, *mecA* detection is important for differentiation of OS-MRSA from MSSA in clinical microbiology laboratory to prevent misdiagnosis. Easy-handling and low-cost systems for *mecA* detection could be a promising strategy for better treatment of MRSA infection.

7. References

1. Gordon RJ, Lowy FD: **Pathogenesis of methicillin-resistant Staphylococcus aureus infection**. *Clin Infect Dis* **46 Suppl 5**:S350-359, 2008.
2. Fluit AC: **Livestock-associated Staphylococcus aureus**. *Clin Microbiol Infect* **18**(8):735-744, 2012.
3. Stapleton PD, Taylor PW: **Methicillin resistance in Staphylococcus aureus: mechanisms and modulation**. *Sci Prog* **85**(Pt 1):57-72, 2002.
4. Boucher HW, Corey GR: **Epidemiology of methicillin-resistant Staphylococcus aureus**. *Clin Infect Dis* **46 Suppl 5**:S344-349, 2008.
5. Cuny C, Wieler LH, Witte W: **Livestock-Associated MRSA: The Impact on Humans**. *Antibiotics (Basel)* **4**(4):521-543, 2015.
6. Foster TJ: **Antibiotic resistance in Staphylococcus aureus. Current status and future prospects**. *FEMS Microbiol Rev* **41**(3):430-449, 2017.
7. Llarrull LI, Fisher JF, Mobashery S: **Molecular basis and phenotype of methicillin resistance in Staphylococcus aureus and insights into new beta-lactams that meet the challenge**. *Antimicrob Agents Chemother* **53**(10):4051-4063, 2009.
8. Fuda CC, Fisher JF, Mobashery S: **Beta-lactam resistance in Staphylococcus aureus: the adaptive resistance of a plastic genome**. *Cell Mol Life Sci* **62**(22):2617-2633, 2005.
9. Mistry H, Sharma P, Mahato S, Saravanan R, Kumar PA, Bhandari V: **Prevalence and Characterization of Oxacillin Susceptible mecA-Positive Clinical Isolates of Staphylococcus aureus Causing Bovine Mastitis in India**. *PLoS One* **11**(9):e0162256, 2016.
10. Saeed K, Ahmad N, Dryden M, Cortes N, Marsh P, Sitjar A, Wyllie S, Bourne S, Hemming J, Jeppesen C, Green S: **Oxacillin-susceptible methicillin-resistant Staphylococcus aureus (OS-MRSA), a hidden resistant mechanism among clinically significant isolates in the Wessex region/UK**. *Infection* **42**(5):843-847, 2014.
11. Andrade-Figueiredo M, Leal-Balbino TC: **Clonal diversity and epidemiological characteristics of Staphylococcus aureus: high prevalence of oxacillin-susceptible mecA-positive Staphylococcus aureus (OS-MRSA) associated with clinical isolates in Brazil**. *BMC Microbiol* **16**(1):115, 2016.
12. Song Y, Cui L, Lv Y, Li Y, Xue F: **Characterisation of clinical isolates of oxacillin-susceptible mecA-positive Staphylococcus aureus in China from 2009 to 2014**. *J Glob Antimicrob Resist* **11**:1-3, 2017.

13. Quijada NM, Hernández M, Oniciuc EA, Eiros JM, Fernández-Natal I, Wagner M, Rodríguez-Lázaro D: **Oxacillin-susceptible mecA-positive Staphylococcus aureus associated with processed food in Europe**. *Food Microbiol* **82**:107-110, 2019.
14. Conceição T, Coelho C, de Lencastre H, Aires-de-Sousa M: **Frequent occurrence of oxacillin-susceptible mecA-positive Staphylococcus aureus (OS-MRSA) strains in two African countries**. *J Antimicrob Chemother* **70**(12):3200-3204, 2015.
15. Hososaka Y, Hanaki H, Endo H, Suzuki Y, Nagasawa Z, Otsuka Y, Nakae T, Sunakawa K: **Characterization of oxacillin-susceptible mecA-positive Staphylococcus aureus: a new type of MRSA**. *J Infect Chemother* **13**(2):79-86, 2007.
16. Institute. CaLS: **CLSI Document. M100-S23.** In.; 2013.
17. Pu W, Su Y, Li J, Li C, Yang Z, Deng H, Ni C: **High incidence of oxacillin-susceptible mecA-positive Staphylococcus aureus (OS-MRSA) associated with bovine mastitis in China**. *PLoS One* **9**(2):e88134, 2014.
18. Sakoulas G, Gold HS, Venkataraman L, DeGirolami PC, Eliopoulos GM, Qian Q: **Methicillin-resistant Staphylococcus aureus: comparison of susceptibility testing methods and analysis of mecA-positive susceptible strains**. *J Clin Microbiol* **39**(11):3946-3951, 2001.
19. Ikonomidis A, Michail G, Vasdeki A, Labrou M, Karavasilis V, Stathopoulos C, Maniatis AN, Pournaras S: **In vitro and in vivo evaluations of oxacillin efficiency against mecA-positive oxacillin-susceptible Staphylococcus aureus**. *Antimicrob Agents Chemother* **52**(11):3905-3908, 2008.
20. Kanesaka I, Fujisaki S, Aiba Y, Watanabe S, Mikawa T, Katsuse AK, Takahashi H, Cui L, Kobayashi I: **Characterization of compensatory mutations associated with restoration of daptomycin-susceptibility in daptomycin non-susceptible methicillin-resistant Staphylococcus aureus and the role mprF mutations**. *J Infect Chemother* **25**(1):1-5, 2019.
21. Wada A, Muratani T, Kobayashi T, Gotoh R, Kido N, Koga K, Matsumoto T: **Ratio of m*ecA* gene in oxacillin-insusceptible and susceptible S*taphylococcus aureus.*** In., vol. 55. Jpn. J. Chemother 374-377, 2007.
22. Chen FJ, Huang IW, Wang CH, Chen PC, Wang HY, Lai JF, Shiau YR, Lauderdale TL, Hospitals T: **mecA-positive Staphylococcus aureus with low-level oxacillin MIC in Taiwan**. *J Clin Microbiol* **50**(5):1679-1683, 2012.
23. Watanabe S, Aiba Y, Tan XE, Li FY, Boonsiri T, Thitiananpakorn K, Cui B, Sato'o Y, Kiga K, Sasahara T, Cui L: **Complete genome sequencing of three**

human clinical isolates of Staphylococcus caprae reveals virulence factors similar to those of S. epidermidis and S. capitis. *BMC Genomics* **19**(1):810, 2018.

24. Watanabe S, Sasahara T, Arai N, Sasaki K, Aiba Y, Sato'o Y, Cui L: **Complete Genome Sequence of Streptococcus pyogenes Strain JMUB1235 Isolated from an Acute Phlegmonous Gastritis Patient**. *Genome Announc* **4**(5) e01133-16, 2016.
25. Watanabe S, Cui B, Kiga K, Aiba Y, Tan X-E, Sato'o Y, Kawauchi M, Boonsiri T, Thitiananpakorn K, Taki Y, Li FY, Azam AH, Nakada Y, Sasahara T, Cui L: **Composition and Diversity of CRISPR-Cas13a systems in the genus Leptotrichia**. *Frontiers in Microbiology* 10:2838, 2019.
26. Gardner SN, Slezak T, Hall BG: **kSNP3.0: SNP detection and phylogenetic analysis of genomes without genome alignment or reference genome**. *Bioinformatics* **31**(17):2877-2878, 2015.
27. Neoh HM, Cui L, Yuzawa H, Takeuchi F, Matsuo M, Hiramatsu K: **Mutated response regulator graR is responsible for phenotypic conversion of Staphylococcus aureus from heterogeneous vancomycin-intermediate resistance to vancomycin-intermediate resistance**. *Antimicrob Agents Chemother* **52**(1):45-53, 2008.
28. Kato F, Sugai M: **A simple method of markerless gene deletion in Staphylococcus aureus**. *J Microbiol Methods* **87**(1):76-81, 2011.
29. Sato'o Y, Aiba Y, Kiga K, Watanabe S, Sasahara T, Hayakawa Y, Cui L: **Optimized universal protocol for electroporation of both coagulase-positive and -negative Staphylococci**. *J Microbiol Methods* **146**:25-32, 2018.
30. Liu P, Xue H, Wu Z, Ma J, Zhao X: **Effect of bla regulators on the susceptible phenotype and phenotypic conversion for oxacillin-susceptible mecA-positive staphylococcal isolates**. *J Antimicrob Chemother* **71**(8):2105-2112, 2016.
31. Kim C, Mwangi M, Chung M, Milheiriço C, Milheirço C, de Lencastre H, Tomasz A: **The mechanism of heterogeneous beta-lactam resistance in MRSA: key role of the stringent stress response**. *PLoS One* **8**(12):e82814, 2013.
32. Pardos de la Gandara M, Borges V, Chung M, Milheiriço C, Gomes JP, de Lencastre H, Tomasz A: **Genetic Determinants of High-Level Oxacillin Resistance in Methicillin-Resistant Staphylococcus aureus**. *Antimicrob Agents Chemother* **62**(6), 2018.

33. Dordel J, Kim C, Chung M, Pardos de la Gándara M, Holden MT, Parkhill J, de Lencastre H, Bentley SD, Tomasz A: **Novel determinants of antibiotic resistance: identification of mutated loci in highly methicillin-resistant subpopulations of methicillin-resistant Staphylococcus aureus**. *MBio* **5**(2):e01000, 2014.
34. Aedo S, Tomasz A: **Role of the Stringent Stress Response in the Antibiotic Resistance Phenotype of Methicillin-Resistant Staphylococcus aureus**. *Antimicrob Agents Chemother* **60**(4):2311-2317, 2016.
35. Bui LM, Conlon BP, Kidd SP: **Antibiotic tolerance and the alternative lifestyles of**. *Essays Biochem* **61**(1):71-79, 2017.
36. Onyango LA, Alreshidi MM: **Adaptive Metabolism in Staphylococci: Survival and Persistence in Environmental and Clinical Settings**. *J Pathog* **2018**:1092632, 2018.
37. Cassels R, Oliva B, Knowles D: **Occurrence of the regulatory nucleotides ppGpp and pppGpp following induction of the stringent response in staphylococci**. *J Bacteriol* **177**(17):5161-5165, 1995.
38. Murakami K, Minamide W, Wada K, Nakamura E, Teraoka H, Watanabe S: **Identification of methicillin-resistant strains of staphylococci by polymerase chain reaction**. *J Clin Microbiol* **29**(10):2240-2244, 1991.
39. Hiramatsu K, Kihara H, Yokota T: **Analysis of borderline-resistant strains of methicillin-resistant Staphylococcus aureus using polymerase chain reaction**. *Microbiol Immunol* **36**(5):445-453, 1992.
40. Kampf G, Adena S, Rüden H, Weist K: **Inducibility and potential role of MecA-gene-positive oxacillin-susceptible Staphylococcus aureus from colonized healthcare workers as a source for nosocomial infections**. *J Hosp Infect* **54**(2):124-129, 2003.
41. Phaku P, Lebughe M, Strauß L, Peters G, Herrmann M, Mumba D, Mellmann A, Muyembe-Tamfum JJ, Schaumburg F: **Unveiling the molecular basis of antimicrobial resistance in Staphylococcus aureus from the Democratic Republic of the Congo using whole genome sequencing**. *Clin Microbiol Infect* **22**(7):644.e641-645, 2016.
42. Duarte FC, Danelli T, Tavares ER, Morguette AEB, Kerbauy G, Grion CMC, Yamauchi LM, Perugini MRE, Yamada-Ogatta SF: **Fatal sepsis caused by mecA-positive oxacillin-susceptible Staphylococcus aureus: First report in a tertiary hospital of southern Brazil**. *J Infect Chemother* **25**(4):293-297, 2019.

43. Roisin S, Nonhoff C, Denis O, Struelens MJ: **Evaluation of new Vitek 2 card and disk diffusion method for determining susceptibility of Staphylococcus aureus to oxacillin**. *J Clin Microbiol* **46**(8):2525-2528, 2008.
44. S S, P S, A K, A. A: **Evaluation of different phenotypic methods for the detection of methicillin resistant Staphylococcus aureus and antimicrobial susceptibility pattern of MRSA**. In., vol. Vol 4. Int J Community Med Public Health; 3297-3301, 2017.
45. Proulx MK, Palace SG, Gandra S, Torres B, Weir S, Stiles T, Ellison RT, Goguen JD: **Reversion From Methicillin Susceptibility to Methicillin Resistance in Staphylococcus aureus During Treatment of Bacteremia**. *J Infect Dis* **213**(6):1041-1048, 2016.
46. Goering RV, Swartzendruber EA, Obradovich AE, Tickler IA, Tenover FC: **Emergence of Oxacillin Resistance in Stealth Methicillin-Resistant**. *Antimicrob Agents Chemother* **63**(8), 2019.
47. Chen FJ, Wang CH, Chen CY, Hsu YC, Wang KT: **Role of the mecA gene in oxacillin resistance in a Staphylococcus aureus clinical strain with a pvl-positive ST59 genetic background**. *Antimicrob Agents Chemother* **58**(2):1047-1054, 2014.
48. Ender M, McCallum N, Berger-Bächi B: **Impact of mecA promoter mutations on mecA expression and beta-lactam resistance levels**. *Int J Med Microbiol* **298**(7-8):607-617, 2008.
49. Aiba Y, Katayama Y, Hishinuma T, Murakami-Kuroda H, Cui L, Hiramatsu K: **Mutation of RNA polymerase β-subunit gene promotes heterogeneous-to-homogeneous conversion of β-lactam resistance in methicillin-resistant Staphylococcus aureus**. *Antimicrob Agents Chemother* **57**(10):4861-4871, 2013.
50. Matsuo M, Yamamoto N, Hishinuma T, Hiramatsu K: **Identification of a Novel Gene Associated with High-Level β-Lactam Resistance in Heterogeneous Vancomycin-Intermediate Staphylococcus aureus Strain Mu3 and Methicillin-Resistant S. aureus Strain N315**. *Antimicrob Agents Chemother* **63**(2), 2019.
51. Mwangi MM, Kim C, Chung M, Tsai J, Vijayadamodar G, Benitez M, Jarvie TP, Du L, Tomasz A: **Whole-genome sequencing reveals a link between β-lactam resistance and synthetases of the alarmone (p)ppGpp in Staphylococcus aureus**. *Microb Drug Resist* **19**(3):153-159, 2013.
52. Bæk KT, Gründling A, Mogensen RG, Thøgersen L, Petersen A, Paulander W, Frees D: **β-Lactam resistance in methicillin-resistant Staphylococcus aureus**

USA300 is increased by inactivation of the ClpXP protease. *Antimicrob Agents Chemother* **58**(8):4593-4603, 2014.

53. Thalsø-Madsen I, Torrubia FR, Xu L, Petersen A, Jensen C, Frees D: **The Sle1 Cell Wall Amidase is essential for β-Lactam Resistance in Community Acquired Methicillin Resistant**. *Antimicrob Agents Chemother* 2019.
54. Lai CC, Chen CC, Lu YC, Chuang YC, Tang HJ: **The clinical significance of silent mutations with respect to ciprofloxacin resistance in MRSA**. *Infect Drug Resist* **11**:681-687, 2018.
55. Ryffel C, Kayser FH, Berger-Bächi B: **Correlation between regulation of mecA transcription and expression of methicillin resistance in staphylococci**. *Antimicrob Agents Chemother* **36**(1):25-31, 1992.
56. Sabat AJ, Pournaras S, Akkerboom V, Tsakris A, Grundmann H, Friedrich AW: **Whole-genome analysis of an oxacillin-susceptible CC80 mecA-positive Staphylococcus aureus clinical isolate: insights into the mechanisms of cryptic methicillin resistance**. *J Antimicrob Chemother* **70**(11):2956-2964, 2015.
57. Anderson KL, Roberts C, Disz T, Vonstein V, Hwang K, Overbeek R, Olson PD, Projan SJ, Dunman PM: **Characterization of the Staphylococcus aureus heat shock, cold shock, stringent, and SOS responses and their effects on log-phase mRNA turnover**. *J Bacteriol* **188**(19):6739-6756, 2006.
58. Hughes J, Mellows G: **On the mode of action of pseudomonic acid: inhibition of protein synthesis in Staphylococcus aureus**. *J Antibiot (Tokyo)* **31**(4):330-335, 1978.
59. Haseltine WA, Block R: **Synthesis of guanosine tetra- and pentaphosphate requires the presence of a codon-specific, uncharged transfer ribonucleic acid in the acceptor site of ribosomes**. *Proc Natl Acad Sci U S A* **70**(5):1564-1568, 1973.
60. Mechold U, Potrykus K, Murphy H, Murakami KS, Cashel M: **Differential regulation by ppGpp versus pppGpp in Escherichia coli**. *Nucleic Acids Res* **41**(12):6175-6189, 2013.
61. Ross W, Vrentas CE, Sanchez-Vazquez P, Gaal T, Gourse RL: **The magic spot: a ppGpp binding site on E. coli RNA polymerase responsible for regulation of transcription initiation**. *Mol Cell* **50**(3):420-429, 2013.
62. Zuo Y, Wang Y, Steitz TA: **The mechanism of E. coli RNA polymerase regulation by ppGpp is suggested by the structure of their complex**. *Mol Cell* **50**(3):430-436, 2013.

63. Krásný L, Gourse RL: **An alternative strategy for bacterial ribosome synthesis: Bacillus subtilis rRNA transcription regulation**. *EMBO J* **23**(22):4473-4483, 2004.
64. Geiger T, Wolz C: **Intersection of the stringent response and the CodY regulon in low GC Gram-positive bacteria**. *Int J Med Microbiol* **304**:150-5, 2014.
65. Pohl K, Francois P, Stenz L, Schlink F, Geiger T, Herbert S, Goerke C, Schrenzel J, Wolz C: **CodY in Staphylococcus aureus: a regulatory link between metabolism and virulence gene expression**. *J Bacteriol* **191**(9):2953-2963, 2009.
66. Shivers RP, Sonenshein AL: **Activation of the Bacillus subtilis global regulator CodY by direct interaction with branched-chain amino acids**. *Mol Microbiol* **53**(2):599-611, 2004.
67. Brinsmade SR: **CodY, a master integrator of metabolism and virulence in Gram-positive bacteria**. *Curr Genet* **63**(3):417-425, 2017.
68. Kaiser JC, King AN, Grigg JC, Sheldon JR, Edgell DR, Murphy MEP, Brinsmade SR, Heinrichs DE: **Repression of branched-chain amino acid synthesis in Staphylococcus aureus is mediated by isoleucine via CodY, and by a leucine-rich attenuator peptide**. *PLoS Genet* **14**(1):e1007159, 2018.
69. Geiger T, Goerke C, Fritz M, Schäfer T, Ohlsen K, Liebeke M, Lalk M, Wolz C: **Role of the (p)ppGpp synthase RSH, a RelA/SpoT homolog, in stringent response and virulence of Staphylococcus aureus**. *Infect Immun* **78**(5):1873-1883, 2010.
70. Andersson DI, Hughes D: **Antibiotic resistance and its cost: is it possible to reverse resistance?** *Nat Rev Microbiol* **8**(4):260-271, 2010.74.
71. Baumert N, von Eiff C, Schaaff F, Peters G, Proctor RA, Sahl HG: **Physiology and antibiotic susceptibility of Staphylococcus aureus small colony variants**. *Microb Drug Resist* 2002, **8**(4):253-260.
72. Chuard C, Vaudaux PE, Proctor RA, Lew DP: **Decreased susceptibility to antibiotic killing of a stable small colony variant of Staphylococcus aureus in fluid phase and on fibronectin-coated surfaces**. *J Antimicrob Chemother* **39**(5):603-608, 1997.
73. Garcia LG, Lemaire S, Kahl BC, Becker K, Proctor RA, Denis O, Tulkens PM, Van Bambeke F: **Antibiotic activity against small-colony variants of Staphylococcus aureus: review of in vitro, animal and clinical data**. *J Antimicrob Chemother* **68**(7):1455-1464, 2013.

74. Cui L, Neoh HM, Iwamoto A, Hiramatsu K: **Coordinated phenotype switching with large-scale chromosome flip-flop inversion observed in bacteria**. *Proc Natl Acad Sci U S A* **109**(25):E1647-1656, 2012.
75. Saito M, Katayama Y, Hishinuma T, Iwamoto A, Aiba Y, Kuwahara-Arai K, Cui L, Matsuo M, Aritaka N, Hiramatsu K: **"Slow VISA," a novel phenotype of vancomycin resistance, found in vitro in heterogeneous vancomycin-intermediate Staphylococcus aureus strain Mu3**. *Antimicrob Agents Chemother* **58**(9):5024-5035, 2014.
76. Katayama Y, Azechi T, Miyazaki M, Takata T, Sekine M, Matsui H, Hanaki H, Yahara K, Sasano H, Asakura K , Takaku T, Ochiai T, Komatsu N, Chambers HF: **Prevalence of Slow-Growth Vancomycin Nonsusceptibility in Methicillin-Resistant Staphylococcus aureus**. *Antimicrob Agents Chemother* **61**(11), 2017.

8. Funding

This work was supported by JMU Graduate Student Start-up Award to TB, JSPS KAKENHI (Grant Nos. 15H05654 and 19K08960 to SW, 18K15149 to KK, 17K15691 to YS, and 17K19570 to LC), the Takeda Science Foundation (LC), and the Japan Agency for Medical Research and Development J-PRIDE (Grant Nos. JP17fm0208028, JP18fm0208028, and JP19fm0208028 to LC). The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

9. **Acknowledgments**

I gratefully acknowledge Prof. Longzhu Cui, Assoc Prof. Shinya Watanabe, Dr. Kotaro Kiga, Dr. Teppei Sasahara, Dr. Yusuke Sato'o, and Dr. Yoshifumi Aiba in Jichi Medical University for the contributions of this research. I would like to gratefully acknowledge Dr. Tetsuro Muratani at Kyurin Medical Laboratory, Dr. Tetsu Mizutani and Ms. Kana Sawa at Osaka Police Hospital, Dr. Tsai-Ling Yang Lauderdale at National Health Research Institutes of Taiwan and Dr. Intetsu Kobayashi at Toho University for kindly providing OS-MRSA strains. I thank Dr. Motoyuki Sugai for kindly gifting plasmids (pKFT and pKAT). I wish appreciate Dr. XinEe Tan, Dr. Moriyuki Kawauchi, Dr. Bintao Cui, Mr. Kanate Thitiananpakorn, Mr. Feng-Yu Li, Mr. R. Narimatsu, Mr. K. Sasaki and Mr. R. Takenouchi in Jichi Medical University who participated in support the laboratory work. I also thank Mrs. Mami Ogawa who helped with general affairs.