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

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ABSTRACT β -Lactam resistance levels vary among methicillin-resistant *Staphylococcus aureus* (MRSA) clinical isolates, mediated by chromosomal mutations and exogenous resistance gene *mecA*. However, MRSA resistance mechanisms are incompletely understood. A P440L mutation in the RNA polymerase β' subunit (RpoC) in slow-vancomycin-intermediate *S. aureus* (sVISA) strain V6-5 is associated with conversion of heterogeneous VISA (hVISA) to sVISA. In this study, we found a V6-5-derivative strain (L4) with significantly decreased MICs to oxacillin (OX) and vancomycin. Whole-genome sequencing revealed that L4 has nonsense mutations in two genes, *relQ*, encoding (p)ppGpp synthetase, an alarmone of the stringent response, and a gene of unknown function. *relQ* deletion in the hVISA strain Mu3 did not affect OX MIC. However, introducing nonsense mutation of the unknown gene into Mu3 decreased OX MIC, whereas wild-type gene recovered high-level resistance. Thus, mutation of this unknown gene (*ehoM*) decreased β -lactam resistance in Mu3 and L4. Presence of *relQ* in a multicopy plasmid restored high-level resistance in strain L4 but not in the *ehoM* mutant Mu3 strain, indicating a genetic interaction between *ehoM* and *relQ* depending on the L4 genetic background. While mupirocin (a stringent response inducer) can increase the β -lactam resistance of MRSA, mupirocin supplementation in an *ehoM* deletion mutant of N315 did not elevate resistance. *ehoM* expression in N315 was induced by mupirocin, and the relative amount of *ehoM* transcript in Mu3 was higher than in N315 induced by the stringent response. Our findings indicate that *ehoM* plays an essential role in high-level β -lactam resistance in MRSA via the stringent response.

KEYWORDS essential gene for resistance, MRSA, oxacillin resistance expression, *relQ* multicopy suppression, stringent response

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a major causative agent of hospital-associated and life-threatening infections (1). In addition to the four common staphylococcal penicillin-binding proteins (PBPs), which synthesize the cell wall peptidoglycan, MRSA produces a fifth PBP, called PBP2a (PBP2') (2–4). In contrast to the native PBPs, PBP2a has an extremely low affinity for β -lactam antibiotics and has been shown to retain its activity even in the presence of concentrations of drugs that inhibit housekeeping PBPs, allowing cell wall biosynthesis to continue (5). PBP2a is encoded by *mecA*, which is carried by a mobile genetic element (staphylococcal cassette chromosome *mec* element [SCC*mec*]) (6, 7).

β -Lactam resistance levels vary among clinical isolates of MRSA (8). This variation in the level of resistance between isolates does not always correlate with the level of PBP2a (3). Early studies using transposon mutagenesis identified several genes that are

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unlinked to *mecA* but that affect β -lactam resistance, including *fem* (factors essential for methicillin resistance), *aux* (auxiliary factors), and *hmt* (high methicillin resistance) (9–13). Many of these genes are involved in cell wall biosynthesis. Due to advancements in sequencing technology, recent studies have revealed many mutations in additional genes associated with high β -lactam resistance of MRSA. Our recent study showed that point mutations in *rpoB*, encoding the RNA polymerase β -subunit, convert low-level to high-level resistance in MRSA strain N315 (14). β -Lactam resistance in a wild-type MRSA strain is heterogeneously expressed. Taking advantage of this feature, Mwangi et al. (15) identified mutations in *relA*, which controls the synthesis of (p)ppGpp, an effector of the stringent response, for homogeneous resistance to β -lactam antibiotics. Moreover, the addition of mupirocin (MUP), an inducer of the stringent response, to the growth medium can convert low-level MRSA to express a highly resistant phenotype (15, 16). Using various clonal types of *S. aureus* strains, it has also been reported that many other mutations in various genes and pathways unlinked to cell wall biosynthesis contribute to mechanisms of resistance to β -lactams (17). However, the resistance mechanisms of MRSA are incompletely understood.

Mu3 is the first clinical isolate identified as a heterogeneous vancomycin (VAN)-intermediate *S. aureus* (hVISA) strain from a patient with VAN treatment failure in Japan in 1997 (18). This isolate's SCC*mec* type and sequence type (SCC*mec* II and ST5) are the same as those of the epidemic MRSA strain N315, which carries the complete *mecA*-regulatory region, *mecR1*, and *mecI* and shows low-level β -lactam resistance (oxacillin [OX] MIC $\leq$ 4 mg/liter) (19). Unlike N315, however, Mu3 shows high-level β -lactam resistance, with an OX MIC of $>1,052$ mg/liter (20), although the mechanism for high-level resistance remains unknown.

We recently reported a new phenotype of VISA, designated slow VISA (sVISA), which survived under VAN pressure, with very slow growth and a higher VAN MIC than extant VISA (21). Expression of this phenotype was very unstable. When passaged on drug-free agar plates, sVISA generated phenotypic revertants that had larger colony sizes and decreased VAN resistance. sVISA strain V6-5 was derived from Mu3, and its phenotype was caused by the P440L mutation in *rpoC*, encoding the RNA polymerase β' subunit. From the analysis of the phenotypic revertants of V6-5, it was suggested that the stringent response is involved in the expression of the sVISA phenotype (22).

In the present study, we found a V6-5 derivative strain, designated L4, with significantly decreased MICs to OX and VAN. To identify the causative mutation, we performed whole-genome analysis of the mutant strain and subsequently assessed the impact of the mutation identified on β -lactam resistance by genetic methods. Moreover, using the deletion mutant, we also investigated the role of the identified gene in high β -lactam resistance induced by the stringent condition.

RESULTS

A functionally unknown gene, SAHV_1047, is essential for the expression of high-level β -lactam resistance in Mu3. Figure 1 shows the results of an OX Etest in strain L4, which has large colony morphology generated from the sVISA strain V6-5. The OX and imipenem (IP) resistance of this strain was dramatically reduced compared with that of its parental sVISA strain (Table 1). The VAN, teicoplanin (TEC), and daptomycin (DAP) MICs of strain L4 also decreased along with those of previously isolated large colony variants (Table 1) (22). PCR analysis revealed that strain L4 retains *mecA* (data not shown), indicating that the decreased β -lactam resistance is caused by additional chromosomal mutation(s) rather than by the deletion of *mecA*. We therefore performed whole-genome sequencing of L4 and its parent strain. Comparative analysis of these two strains using the Mu3 complete genome sequence (20) revealed that L4 harbors two nonsense mutations in the N-terminal domains of two genes, *relQ*, encoding ppGpp synthetase, and gene SAHV_1047, whose function is unknown (Table 1). As this result depicts that these two genes lose their function, we first performed a complementation test to identify which mutation is responsible for the decreased OX resistance. Introducing the wild-type SAHV_1047 gene into strain L4 using a multicopy

FIG 1 Essentiality of *ehoM* (SAHV_1047) for high β -lactam resistance in Mu3 and strain L4-specific *relQ* multicopy suppression of decreased OX resistance caused by *ehoM*. Etest for OX was performed using a BHI agar plate. The incubation time for each plate is shown on the left. (A) L4/pSR, L4/pSR_1047, L4/pSR_1047, and V6-5; (B) Mu3, Mu3 Δ *relQ*, and Mu3_1047*; (C) Mu3_1047*/pSR, Mu3_1047*/pSR_1047, and Mu3_1047*/pSR_1047.

plasmid recovered the decreased OX MIC from 2 to >256 mg/liter after a 24-h incubation (Fig. 1A). While the introduction of *relQ* with pSR_1047 into L4 raised the level of OX resistance, it did not reach the parental level after a 24-h incubation (Fig. 1A). Therefore, we consider that the mutations in SAHV_1047 caused the decreased OX resistance.

To confirm this result, we introduced a nonsense mutation (K8*) of SAHV_1047 found in L4 into the parental strain Mu3 using an allelic-replacement strategy. The resultant strain, Mu3_1047*, showed a substantially decreased OX MIC, from >256 mg/liter to 4 mg/liter (Fig. 1B). Moreover, the decreased resistance was recovered to the resistance level of Mu3 by the introduction of the wild-type SAHV_1047 gene (Fig. 1B and C). In contrast, deletion of the *relQ* gene in Mu3 did not affect OX resistance (Fig. 1B). These results clearly demonstrate that the nonsense (K8*) mutation in SAHV_1047 is associated with the decrease in high-level β -lactam resistance in L4 and Mu3. We named this novel gene *ehoM* (essential gene for high-level oxacillin resistance in Mu3).

TABLE 1 Antibiogram and mutations of L4 and its parental strains

Strain	Phenotype	MIC (mg/liter) ^a						Mutation(s)
		OX	IP	VAN	TEC	DAP	MU	
Mu3 <i>fdh2</i> *	hVISA	>256	>32	3	12	3	0.064	
V6-5	sVISA	>256	>32	16	24	4	0.064	<i>rpoC</i> (P440L)
L4	VISA	2	0.64	8	8	3	0.19	<i>rpoC</i> (P440L), <i>relQ</i> (Y29*), SAHV_1047(R3S, K8*)

^aMIC was determined using Etest after a 72-h incubation on a brain heart infusion agar plate.

TABLE 2 MICs of β -lactams and whole-genome sequences in *relQ* multicopy suppressor mutants

Strain	OX MIC (mg/liter) ^a	RpoC mutation		Etest plate used for isolation	Sequencing region
		Amino acid change	Nucleotide sequence		
L4/pSR (parent)	1.5	P440L	CTA		ND ^c
L4/pSR_1047	>256	NC ^b	CTA	OX	Whole genome
L4/pSR_relQ-OR1	>256	L440S	TCA	OX	Whole genome
L4/pSR_relQ-OR2	>256	L440S	TCA	OX	Whole genome
L4/pSR_relQ-OR3	>256	L440S	TCA	OX	Whole genome
L4/pSR_relQ-OR4	>256	L440P	CCA	OX	Whole genome
L4/pSR_relQ-OR5	>256	L440S	TCA	OX	<i>rpoC</i>
L4/pSR_relQ-OR6	>256	L440V	GTA	OX	<i>rpoC</i>
L4/pSR_relQ-OR7	>256	NC	CTA	OX	<i>rpoC</i>
L4/pSR_relQ-OR8	>256	L440V	GTA	OX	<i>rpoC</i>
L4/pSR_relQ-IR-1	>256	NC	CTA	IP	<i>rpoC</i>
L4/pSR_relQ-IR-2	>256	L440S	TCA	IP	<i>rpoC</i>
L4/pSR_relQ-IR-3	>256	L440P	CCA	IP	<i>rpoC</i>

^aMIC was determined using the Etest after a 48-h incubation.^bNC, not changed.^cND, not determined.

and its derivative strains) and further characterized the roles of this gene in OX resistance.

***relQ* multicopy suppression of decreased β -lactam resistance caused by the *ehoM* mutation depends on the L4 genetic background.** To our surprise, after a 48-h incubation, the introduction of *relQ* in multicopy to strain L4 also returned the OX resistance, with an MIC of >256 mg/liter (Fig. 1A). However, the colony morphology was apparently different from that of strain L4/pSR_1047 (Fig. 1A). Therefore, we suspected that the presence of *relQ* in multicopy suppresses the depression of high resistance caused by deletion of the *ehoM* gene in MRSA. To test this hypothesis, we introduced pSR_relQ into the *ehoM* nonsense mutant strain Mu3_1047* and measured MIC of OX in the resultant strain, Mu3_1047*/pSR_relQ. However, such multicopy suppression with *relQ* was not observed in this strain (Fig. 1C). These results indicate a genetic interaction dependent on the L4 genetic background between *ehoM* and *relQ*.

To further clarify the L4-specific genetic interaction between *relQ* and *ehoM*, we picked four OX-resistant colonies of L4/pSR_relQ that appeared on the inhibitory zone of the Etest plates (Fig. 1A) and performed whole-genome sequencing; we selected L4/pSR_1047 as a control. The comparative analysis of L4 and the resultant sequencing revealed that all four strains, L4/pSR_relQ-OR1 to -OR4, have only a single mutation in *rpoC*, where the leucine at position 440 was changed to serine in three strains and proline in one strain (Table 2). In contrast, L4/pSR_1047 had no mutation in any open reading frame (ORF). Moreover, another four OX-resistant colonies from other OX Etest plates and three IP-resistant colonies from an IP Etest plate were picked, and *rpoC* was sequenced. Sequencing results indicated that the leucine at the same position was changed to serine in two strains, valine in two strains, and proline in one strain and that there was no change in *rpoC* in two other strains (Table 2). All these isolates showed increased OX MICs (Table 2). These results suggest that there are two mechanisms regulating the *relQ* multisuppression of β -lactam-resistant depression in L4: *rpoC*(P440L)-dependent and *rpoC*(P440L)-independent mechanisms.

***ehoM* plays an essential role in high β -lactam resistance induced by the stringent response.** *relQ* encodes a synthetase of (p)ppGpp, a molecular alarmone of the stringent response (23). MUP is an inducer of the stringent response (24, 25) and can convert low-level β -lactam resistance to high-level resistance in MRSA strains (16). Based on the above-described observation of the genetic interaction, we investigated the effect of the stringent response using MUP on OX resistance in L4. The OX MIC of L4 in the presence of sub-MIC MUP (0.03 mg/liter) was the same as that in the absence of sub-MIC MUP (6 mg/liter), and the MIC did not show the same high resistance level as that of the parent strain V6-5 (>256 mg/liter) (Table 3). The addition of MUP did not lead to high-level resistance in either L4 or Mu3_1047* (Table 3). Mu3 is highly resistant

TABLE 3 Effects of *ehoM* and *relQ* on OX MICs in the presence or absence of MUP

Strain	OX MIC (mg/liter) ^a	
	Without MUP	With MUP ^b
V6-5	>256	>256
L4	6	6
Mu3	>256	>256
Mu3_1047*	8	12
Mu3Δ <i>relQ</i>	>256	>256
Mu3_1047*/pSR	8	8
Mu3_1047*/pSR_1047	>256	>256
Mu3_1047*/pSR_Δ <i>relQ</i>	24	12
N315	6	256
N315Δ1047	1.5	3
N315Δ <i>relQ</i>	6	>256
N315/pSR	6	256
N315/pSR_1047	6	256
N315/pSR_Δ <i>relQ</i>	32	256

^aMICs were determined using the Etest on a cation-adjusted MH agar plate after a 24-h incubation according to the manufacturer's instructions. However, the MIC for L4 was determined after 48 h because its growth was too slow to measure the MIC in the presence of MUP. The OX MIC of L4 after a 24-h incubation was 1 mg/liter.

^bThe agar plate contained 0.03 mg/liter of MUP.

to β -lactams without MUP; the OX MIC of Mu3 is >256 mg/liter (Table 3) (20). Therefore, it may be that the contribution of *ehoM* to resistance is restricted to the background of Mu3. To clarify this point, we also examined the effect of the MRSA strain N315 (OX MIC = 6 mg/liter), in which provocation of the stringent response has been shown to result in the conversion of low-level to high-level β -lactam resistance (16, 26). As shown in Table 3, the deletion of *ehoM* in N315 (N315Δ1047) decreased OX MIC from 6 to 1.5 mg/liter. Upon supplementation of sub-MIC with MUP (0.03 mg/liter), the OX MIC of N315Δ1047 did not significantly change (3 mg/liter) but remained at the same level as that of the parental strain without MUP (Table 3). These results indicate that *ehoM* plays an essential role in high β -lactam resistance in not only Mu3 but also N315 under the stringent condition.

Role of *relQ* in high-level β -lactam resistance. Next we investigated the role of *relQ* in high-level β -lactam resistance. Deletion of *relQ* in Mu3 retained an OX MIC of >256 mg/liter, indicating that *relQ* is unnecessary for the expression of high-level OX resistance in Mu3 (Fig. 1B and Table 3). The *relQ* deletion in strain N315 did not affect the OX MIC, and the MIC value in N315Δ*relQ* in the presence of MUP increased to >256 mg/liter, which is the same level as for the parental strain induced by the supplementation of MUP (Table 3). These results indicate that *relQ* is not necessary for the provocation of high-level β -lactam resistance in N315.

Next, we introduced plasmid pSR_Δ*relQ* into N315. The resultant strain, N315/pSR_Δ*relQ*, raised the OX MIC from 6 to 32 mg/liter (Table 3). *S. aureus* possesses another two (p)ppGpp synthases, RelA and RelP, in addition to RelQ. The involvement of *relA* in OX high-level resistance has been demonstrated previously (15). In addition, both RelQ and RelP function as (p)ppGpp synthetases that are induced under cell envelope stress (23). Hence, one can infer the functional redundancy among these genes as the reason why the *relQ* deletion mutant strains did not express the phenotype, despite the fact that the stringent condition induces high-level β -lactam resistance. Not only *relQ* but also other (p)ppGpp synthases, complementarily functioning, are possibly involved in high antibiotic resistance in N315.

Expression of *ehoM* induced by mupirocin and increased expression of *ehoM* in Mu3. To elucidate the role of *ehoM* in OX resistance, we next analyzed *ehoM* gene expression using a quantitative real-time PCR (qRT-PCR) assay. The addition of OX to the growth medium did not significantly impact the relative quantification (RQ) of N315 (Fig. 2). When a subinhibitory concentration of MUP was added, however, the relative amount of the *ehoM* transcript in strain N315 increased 2.0-fold ($P < 0.05$) (Fig. 2). These

FIG 2 Relative expression of *ehoM* by qRT-PCR in N315, Mu3, and Mu3_1047* in the presence or absence of OX, MUP, or both OX and MUP. Relative transcript levels (RQ) of *eho* represent the means ($\pm$ SDs) of three biological replicates *in vitro*. The fold changes represent *eho* expression normalized to the housekeeping gene *gyrB*. *, $P < 0.05$; **, $P < 0.001$ (compared with each strain in the absence of any drug).

results imply that the expression of *ehoM* in N315 is induced by the stringent response rather than by β -lactam. In contrast, such induction by MUP was not observed in Mu3. However, the RQ of the *ehoM* transcript in Mu3 was higher than that of N315, approximately 3.3-fold ($P < 0.05$) (Fig. 2). In Mu3, the supplementation of OX resulted in a <2 -fold increase in the RQ of *ehoM*.

Since Mu3_1047* is a nonsense, not deletion, *ehoM* mutant, it is possible to assess the expression level of the *ehoM* transcript in this strain. As shown in Fig. 2, there was no significant difference in the gene expression levels between Mu3 and Mu3_1047* in the absence of drugs and in the presence of either OX or MUP or both.

Effect of *ehoM* on *mecA* gene expression. To examine the effect of *ehoM* on *mecA* expression, we performed qRT-PCR assay of the *mecA* transcript in N315 Δ 1047 and its parent strain. As shown in Fig. 3A, the expression of *mecA* in N315 was upregulated by

FIG 3 Effect of *ehoM* on *mecA* expression and the amount of PBP2a in N315 Δ 1047 and N315. (A) Relative expression of *mecA* in N315 Δ 1047 and N315 in presence or absence of OX, MUP, or OX and MUP. Relative transcript levels of *mecA* represent the means ($\pm$ SDs) of three biological replicates *in vitro*. The fold changes represent *mecA* gene expression ($2^{-\Delta\Delta CT}$) to the housekeeping gene *gyrB*. *, $P < 0.05$; **, $P < 0.001$ (compared with N315 strain in the absence of drug). (B) Relative amounts of PBP2a in N315 Δ 1047 and N315 in presence or absence of OX, MUP, or OX and MUP by Western blotting.

FIG 4 Effect of *ehoM* on the *mecA* expression and the amount of PBP2a in Mu3 and Mu3_1047*. (A) Relative expression of *mecA* in Mu3 and Mu3_1047* in the presence or absence of OX. Relative transcript levels of *mecA* represent the mean ($\pm$ SDs) of three biological replicates *in vitro*. The fold changes represent *mecA* gene expression ($2^{-\Delta CT}$) normalized to the housekeeping gene *gyrB*. *, $P < 0.05$; **, $P < 0.001$ (compared with N315 strain in the absence of any drug). (B) Relative amounts of PBP2a in Mu3 and Mu3_1047* in the presence or absence of OX by Western blotting.

the addition of MUP or OX. When OX in the presence of MUP was added, RQ of *mecA* in N315 showed a 17-fold increase, a finding previously described by Aedo and Tomasz (26). The same upregulations were also observed in N315 Δ 1047, and there was no significant difference in RQ of the *mecA* transcript between these two strains, indicating that *ehoM* does not play a regulatory role in *mecA* transcription.

It was observed that the levels of both the *mecA* transcript and the PBP2a protein increased in the N315 cells under the stringent condition (26). To test the effect of *ehoM* on the amount of PBP2a, we next compared the relative amounts of PBP2a between N315 Δ 1047 and N315 by Western blotting. As shown in Fig. 3B, the Western blot analysis revealed that there was no decrease in the relative amount of PBP2a in the *ehoM* deletion N315 mutant. Therefore, the lack of high OX resistance induced by MUP in N315 Δ 1047 cannot be explained by the decrease in the amount of PBP2a.

We next explored the impact of *ehoM* in Mu3 on *mecA* expression. The basal expression level of *mecA* in both Mu3 and Mu3_1047* was 100-fold higher than that of N315, and there was no significant difference in RQ of the *mecA* transcript between Mu3 and Mu3_1047* (Fig. 4A). The RQ of the *mecA* transcript in Mu3 was not changed by the addition of OX. Although the expression of *mecA* in Mu3_1047* was downregulated in the presence of OX, the range was less than 2-fold (Fig. 4A). Western blot analysis revealed that there was no significant difference in the relative amounts of PBP2a of the *ehoM* deletion Mu3 mutant strain in the presence and absence of OX (Fig. 4B). From these results, *ehoM* does not appear to be involved in the expression of PBP2a.

Dissemination of EhoM in the genus *Staphylococcus* and others. *ehoM* encodes a protein of unknown function comprising 156 amino acid residues, found in the Pfam database under accession number DUF2538 (<http://pfam.xfam.org/>). To determine the conservation of EhoM in bacteria, the EhoM amino acid sequence was analyzed using BLAST (27). Further, $\geq 40\%$ identity over the entire protein sequence was chosen as a cutoff. The comparison was restricted to species with available genome sequences. The results revealed that genes homologous to *ehoM* are found in all members of *Staphylococcus* except *S. cohnii* and in only some Gram-positive bacteria, such as *Macrococcus*.

DISCUSSION

In the present study, we identified a novel gene, designated *ehoM*, which is essential for high-level β -lactam resistance in the hVISA clinical strain Mu3. Moreover, *relQ* was identified as a multicopy suppressor of the *ehMo*-defective phenotype depending on the L4 genetic background.

L4, as well as its parent strain V6-5, harbors two mutations: *rpoC*(P440L) and *fdh2*(A297V), which are not detected in Mu3 (22, 28). Whole-genome analysis of the *relQ*

multicopy suppressors in the decreased β -lactam resistance phenotype of L4 revealed that there are at least two mechanisms at work here: *rpoC*(P440L)-dependent -independent pathways (Table 2). Our previous study suggested that the *rpoC*(P440L) mutation caused the sVISA phenotype through the stringent response via *relQ* (22). As such, the stringent condition in L4 might differ from that in *rpoC* and/or *fdh2* wild-type strains, causing *relQ* multicopy suppression of the *ehoM*-defective phenotype.

Mu3 harbored two single nucleotide polymorphism (SNPs) in *relA*: R117H, found in the (p)ppGpp hydrolase domain, and A567E, found between the TGS and ACT domains. In addition to *relA*, another SNP in Mu3 was found in *relQ* (20). These SNPs in *relA* and *relQ* have not been detected in other *S. aureus* strains that have already been sequenced, including N315, with the exception of the VISA strain Mu50, which is closely related to Mu3. Both strains Mu3 and Mu50 exhibit a slow-glowing phenotype, which may be caused by a constitutive active stringent condition resulting from the accumulation of (p)ppGpp. It has been reported that a point mutation found in the hydrolase domain of *relA* in the VISA clinical strain JKD6229 causes RelA hydrolase activity and constitutive activation of the stringent response (29).

We also demonstrated in the present study that *ehoM* plays an essential role in high-level OX resistance induced by the stringent response in N315, based on susceptibility experiments using an *ehoM* deletion mutant (Table 3). Furthermore, qRT-PCR analysis revealed that *ehoM* gene expression in N315 is induced by MUP, and the amount of the *ehoM* transcript in Mu3 was higher than that in N315 under the stringent condition (3.3-fold; $P < 0.05$). The addition of MUP did not significantly impact the RQ of Mu3 (Fig. 3). These observations suggest a possible mechanism underlying high β -lactam resistance in Mu3; while being exposed to various stress stimuli, including β -lactam antibiotic, Mu3 acquired mutations in (p)ppGpp synthetase genes, such as *relA* and *relQ*, which could be consecutively inducible under stringent conditions. Such mutations resulted in the upregulation of *ehoM* expression, leading to high resistance.

An increase in the amount of PBP2a in cells contributes to the increased β -lactam resistance caused by the stringent stress (26). However, in this study, we did not observe such decreases in both the expression of *mecA* and the amount of PBP2a in the *ehoM* deletion mutant strain compared with the wild-type strain N315 (Fig. 3). Similarly, in the Mu3 background, the deletion of *ehoM* did not significantly change the amount of PBP2a (Fig. 4B). What mechanism does *ehoM* contribute to the high resistance of MRSA? One reasonable explanation is the involvement of *ehoM* in the reduction of PBP2 activity. Recently, it was reported that PBP4 is involved in the high resistance against β -lactams (30). Accordingly, another possible mechanism is that *ehoM* may contribute to the resistance by affecting a PBP protein other than PBP2a, such as PBP4.

Finally, our BLAST analysis revealed that genes homologous to *ehoM* are found in all *S. aureus* strains and only some Gram-positive bacteria. Therefore, *ehoM* might become an effective target of an anti-MRSA agent.

This study provides direct evidence of the essentiality of *ehoM* for high β -lactam resistance in Mu3. Moreover, our study reveals the necessity of *ehoM* for the high β -lactam resistance in N315 induced by a stringent response inducer. Further investigations of the effect of *ehoM* on PBP2a function or the expression of other PBP proteins along with direct measurement of the level of (p)ppGpp in Mu3 can facilitate a better understanding of the mechanism of high-level resistance in MRSA.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Bacterial strains and plasmids used in the present study are listed in Table 4. *S. aureus* strains were grown in brain heart infusion (BHI) broth or agar with aeration at 37°C unless indicated otherwise.

Isolation of L4 from *rpoC*(P440L) sVISA strain V6-5. A total of 10^5 sVISA cells were inoculated into 4 ml of BHI broth. After 20 h of cultivation at 37°C, a portion of the culture containing approximately 10^3 CFU was spread onto BHI agar plates. Single large colonies were then picked from plates after a 24-h incubation. A total of 11 colonies were isolated and examined for OX resistance. Of the 11 colonies, only one strain, named L4, showed decreased OX resistance and was used in further studies.

Recombinant DNA techniques. Extraction and purification of plasmid DNA from *Escherichia coli*, DNA isolation from *S. aureus* cells, restriction endonuclease digestion, ligation reactions, and DNA cloning

TABLE 4 Strains and plasmids used in this study

Strain or plasmid	Description	Reference
Strains		
Mu3	hVISA clinical isolate	18
Mu3 <i>fdh2</i> *	hVISA; Mu3-derived construct with <i>fdh2</i> replaced by <i>fdh2</i> (A297V)	30
V6-5	<i>rpoC</i> (P440L) mutant sVISA strain obtained by selection of Mu3 <i>fdh2</i> * with 6 mg/liter of VAN	22
L4	Derivative of V6-5 with large-colony morphology and decreased β -lactam antibiotics	This study
Mu3 Δ <i>relQ</i>	Mu3-derived <i>relQ</i> deletion construct	This study
Mu3_1047*	Mu3-derived construct with SAVH_1047 replaced by SAVH_1047(K8*)	This study
N315	Pre-MRSA clinical isolate	
N315 Δ <i>relQ</i>	N315-derived <i>relQ</i> deletion construct	This study
N315 Δ 1047	N315-derived SAVH_1047 deletion construct	This study
Plasmids		
pSR	Multicopy <i>E. coli</i> - <i>S. aureus</i> shuttle vector pKE515	31
pSR_1047	pKE515 carrying wild-type (Mu3 genotype) <i>relQ</i>	22

were performed as described previously (30). To construct plasmid pSR_1047, SAVH_1047 (*ehoM*) of Mu3 chromosomal DNA was amplified using primers 1047-CP1 and 1047-CP2 (see Table S1 in the supplemental material) and was inserted into the BamHI and KpnI sites of the pKE515 plasmid vector (28). The integrity of the cloned SAVH_1047 was determined by sequencing the recombinant plasmid using primers pSR-1 and pSR-2 (22). The resultant pSR_1047 and pSR_1047 (*relQ*) plasmids were then introduced into L4, Mu3_1047*, and N315.

Construction of gene replacement and mutant strains. The pKOR1 allele replacement system was used to construct gene replacement and deletion mutants as described previously (31). To replace *ehoM* in Mu3 with the nonsense point mutation (K8*) found in L4, a DNA fragment of approximately 1.0 kb containing SAVH_1047 was amplified by PCR using attB1_1047, whose sequence was designed to generate the K8* mutation, and attB2_1047 primers with Mu3 genomic DNA was used as the template. The primer sequences are listed in Table S1 in the supplemental material. For the deletion of *ehoM* and *relQ*, up- and downstream regions of each gene were amplified by PCR using the primer sets listed in Table S1 and genomic DNA of Mu3 as the template. The PCR product sets of each gene were then ligated by joining PCR. The resultant PCR product with the *attB* site at both ends was used for recombination with pKOR1, yielding plasmids pKOR_1047*, pKOR Δ 1047, and pKOR Δ *relQ*. Sequence integrity of the constructs was confirmed by sequencing using the primers listed in Table S1. These plasmids were then used in the allelic replacement procedure (32).

Antimicrobial susceptibility testing. MICs were determined using the Etest (AB Biodisk, Solna, Sweden) in accordance with the manufacturer's recommendations. For examining the essentiality of *ehoM* and performing multicopy suppression experiments, BHI agar was used.

Whole-genome sequencing and identification of mutations. DNA extraction was performed using a Qiagen genome-tip system (Qiagen), and library preparations were performed using Nextera XT DNA sample preparation kits (Illumina, Inc., San Diego, CA) in accordance with the manufacturer's recommendations. Samples were sequenced on the Illumina MiSeq platform, and 250-bp end reads were generated. Reads from bacterial strains were then mapped to the reference whole-genome sequence of the Mu3 strain (GenBank accession no. AP009324.1), and mutations were identified using CLC Genomics Workbench version 8.5 (CLC bio).

Measurement of *ehoM* and *mecA* gene expressions by quantitative RT-PCR analysis. RNA was extracted from exponentially growing *S. aureus* cells (optical density at 600 nm [OD₆₀₀] = 1) using an RNeasy minikit (Qiagen, Gaithersburg, MD). RNA was reverse transcribed to cDNA using the SuperScript IV VLO reverse transcriptase system (Invitrogen by Thermo Fisher Scientific) according to the manufacturer's instructions. Quantitative real-time PCR was performed using a cDNA template, Fast SYBR green master mix (Applied Biosystems), and primers (Table S1). The signals were detected by the ABI 7500 FAST real-time PCR system (Applied Biosystems). All samples were tested in triplicate in three independent experiments. Transcript levels were normalized to the level of the endogenous control gene, *gyrB* (33), and relative target gene expression was calculated according to the comparative *C_T* method ($\Delta\Delta C_T$ method) as described elsewhere (34). Data are presented as the fold change in transcript levels compared to those of the N315 strain in the absence of any drug.

Preparation of staphylococcal membrane proteins. Membrane fractions were prepared from the *S. aureus* strains following the method described previously (35), with modifications. Briefly, to determine the amounts of PBP2a associated with the bacterial plasma membrane, *S. aureus* strains N315, N315 Δ 1047, Mu3, and Mu3_1047* were grown at 37°C in 200 ml of BHI. MUP (0.03 μ g/ml) and/or OX (1 μ g/ml) was added to the growth media to test the effects of these agents on the amounts of PBP2a produced by the bacteria. All strains were harvested at an OD₆₀₀ of 1.0, washed, and resuspended in 3 ml of 20 mM Tris-HCl, pH 7.6, containing 1 $\times$ Halt protease inhibitor cocktail (Thermo Fisher Scientific, Inc.), 10 mM MgCl₂, 100 μ g/ml of lysostaphin, 50 μ g/ml of lysozyme, 50 μ g/ml of DNase I, and 50 μ g/ml of RNase A. Cells were incubated at 37°C for 30 min and disrupted by sonication with a pulse of 40% output for 5 min. The suspensions were centrifuged at 7,000 $\times g$ for 20 min to remove unbroken debris, and the supernatants were transferred to fresh ultracentrifuge tubes. Membrane fractions were collected by centrifugation at 100,000 $\times g$ for 1 h. The collected membranes were resuspended in 20 mM Tris-HCl, pH

7.6, and stored at -70°C . The concentration of total membrane protein was determined using the 660-nm protein assay (Thermo Fisher Scientific, Inc.).

Western blotting and CBB staining. Western blotting was performed to detect PBP2a in membrane protein. One microgram of membrane protein was loaded onto SDS-PAGE gels for Western blotting. The primary antibody (mouse monoclonal anti-PBP2a antibody, a kind gift from Denka Seiken) was used at a dilution of 1:2,000, and the secondary antibody was horseradish peroxidase (HRP)-coupled goat anti mouse IgG antibody (2 mg/ml; Abcam; ab205719), which was diluted 1:2,000. Amersham ECL Prime (GE Healthcare) was used for the HRP substrate to visualize PBP2a bands with chemiluminescence, which were scanned on an ImageQuant LAS4000 system (GE Healthcare). For Coomassie brilliant blue (CBB) staining, 20 μg of the membrane protein was loaded onto the SDS-PAGE gels. After electrophoresis, the gels were stained with CBB Stain One Super (Nacalai tesque) according to the manufacturer's instructions.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AAC.00712-18>.

SUPPLEMENTAL FILE 1, XLSX file, 0.02 MB.

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