The NaHCO₃-Responsive Phenotype in Methicillin-Resistant
Staphylococcus aureus (MRSA) Is Influenced by mecA Genotype

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ABSTRACT
Methicillin-resistant Staphylococcus aureus (MRSA) strains are a leading cause of many invasive clinical syndromes, and pose treatment difficulties due to their in vitro resistance to most β-lactams on standard laboratory testing. A novel phenotype frequently identified in MRSA strains, termed ‘NaHCO₃-responsiveness’, is a property whereby strains are susceptible in vitro to many β-lactams in the presence of NaHCO₃. Specific mecA genotypes, repression of mecA/PBP2a expression and perturbed maturation of PBP2a by NaHCO₃ have all been associated with this phenotype. The aim of this study was to define the relationship between specific mecA genotypes and PBP2a substitutions, on the one hand, with NaHCO₃-responsiveness in vitro. Mutations were made in the mecA ribosomal binding site (RBS -7) and at amino acid position 246 of its coding region in parental strains MW2 (NaHCO₃-responsive) and C36 (NaHCO₃- nonresponsive) to generate ‘swap’ variants, each harboring the other’s mecA-RBS/coding region genotypes. Successful swaps were confirmed by both sequencing, as well as predicted swap of in vitro penicillin-clavulanate susceptibility phenotypes. MW2 swap variants harboring the nonresponsive mecA genotypes became NaHCO₃-nonresponsive (resistant to the β-lactam, oxacillin [OXA]), in the presence of NaHCO₃. Moreover, these swap variants had lost NaHCO₃-mediated repression of mecA/PBP2a expression. In contrast, C36 swap variants harboring the NaHCO₃-responsive mecA genotypes remained NaHCO₃-nonresponsive phenotypically, and still exhibited nonrepressible mecA/PBP2a expression. These data demonstrate that in addition to the mecA genotype, NaHCO₃-responsiveness may also depend on strain-specific genetic backgrounds.

KEYWORDS
Methicillin-resistant Staphylococcus aureus (MRSA), β-lactam, sodium bicarbonate (NaHCO₃), mecA, penicillin-binding protein 2a (PBP2a)
including for MRSA (8–14). These investigations have led to the discovery of a novel phenotype in a rather large proportion of MRSA strains, termed "NaHCO₃-responsiveness" (9, 10, 15). NaHCO₃-responsive strains display a substantial (≥4-fold) reduction in their in vitro MICs for certain β-lactams (usually into the ‘susceptible’ MIC range) when grown in the presence of NaHCO₃ (10, 15). Moreover, NaHCO₃-responsive MRSA strains are effectively cleared by β-lactam therapy in both ex vivo and in vivo experimental endocarditis models (10).

This phenotype is multifactorial, but features perturbations in: (i) expression of mecA, which encodes the alternative penicillin-binding protein (PBP) 2a, the primary determinant of β-lactam resistance in S. aureus (16–19); and (ii) other genes necessary for the proper functioning of PBP2a in carrying out cell wall biosynthesis (10, 20, 21). PBP2a functions via allosteric regulation of the active binding site for peptidoglycan precursors, which remains “closed” in the presence of traditional β-lactams (22–24). In contrast, newer generation cephalosporins (ceftaroline; ceftabiprole) are able to open such sites (25, 26).

Recently, another unique β-lactam susceptibility phenotype in MRSA was identified by Harrison et al., wherein strains possessing distinct mecA genotypes were found to be either ‘susceptible’ or ‘resistant’ to a combination of β-lactam/β-lactamase inhibitors (27). These investigations identified specific regions within mecA of particular importance to this phenotype, including both the upstream -7 site, corresponding to the mecA promoter/ribosomal binding site (RBS) region (28), as well as the 246th amino acid position of PBP2a (27). Alteration of the promoter/RBS sequence was found to alter expression of mecA, while substitution at the 246th amino acid position was found to alter penicillin binding to PBP2a in the presence of clavulanic acid (27). Thus, specific mecA genotypes, with respect to these two loci, have been dubbed ‘susceptible’ or ‘resistant’ in relation to the ability of β-lactam/β-lactamase inhibitors to inhibit such strains (27).

The aims of the current study were to determine the impacts of particular ‘susceptible’ and ‘resistant’ mecA genotypes (as per Harrison et al. [27]), on the one hand, with the NaHCO₃-responsiveness phenotype, on the other hand. To this end, we constructed isogenic mutants with various ‘susceptible’ and ‘resistant’ mecA genotypes, as defined above (27), in prototype NaHCO₃-responsive and nonresponsive MRSA strain backgrounds. These mutant strains were then assessed for both ‘swap’ of their susceptibility to β-lactams/β-lactamase inhibitors, as well of their NaHCO₃-responsiveness phenotypes. Additionally, the impact of these mutational swaps on mecA transcription, translation, and PBP2a protein production/localization was determined.

RESULTS

Impact of mecA alleles on susceptibility to β-lactams. To determine the impact of altered mecA RBS and/or coding sequences on susceptibility to β-lactams, isogenic mutants were constructed in the MW2 (NaHCO₃-responsive) and C36 (nonresponsive) parental backgrounds harboring chromosomal alterations at the mecA RBS -7 site and/or the 246th amino acid position in PBP2a. The NaHCO₃-responsiveness phenotype is defined by ≥4-fold increased susceptibility to β-lactam antibiotic in the presence of NaHCO₃ (44 mM) compared to its absence (10). As above, the specific point mutations introduced were originally designated being determinative of “susceptibility” or “resistance” to penicillin in the presence of clavulanic acid (27). Specifically, strains harboring the RBS -7T allele (with either 246E or 246G amino acid) were determined to be susceptible to the combination of penicillin (PEN) plus clavulanic acid (CLAV); strains harboring the RBS -7G allele (with either 246E or 246G amino acid) were resistant to this β-lactam/β-lactamase combination (27). Assessment of the impact of these genetic alterations in the MW2 and C36 strain backgrounds revealed that swap mutants harboring the RBS -7G allele were more resistant to penicillin plus clavulanic acid by Etest than strains harboring the RBS -7T allele in both backgrounds (Table 1), confirming prior reports (27–29).

The parental responsive MW2 and nonresponsive C36 strains (10, 15, 30) and their swap constructs were then assessed for susceptibility to the anti-staphylococcal β-lactam, oxacillin (OXA), in the presence or absence of NaHCO₃. Upon minimum inhibitory concentration (MIC) assessment of the MW2 parent and its mecA swap mutants, alteration of the RBS -7 site
| Strain            | Allele | Oxacillin MIC (µg/mL) | PEN + CLAV MIC (µg/mL) | NaHCO₃ impact on: | meCA gene expression | PBP2a protein production |
|-------------------|--------|-----------------------|------------------------|------------------|----------------------|------------------------|
|                   |        | CA-MHB               | CA-MHB tris            | 44 mM NaHCO₃     |                      |                        |
| MW2 parent        | T      | 44                 | 2                      | 0.38 (S)         | Decrease             | Decrease               |
| ALC9188           | T      | 4                  | 8                      | 0.25 (S)         | Decrease             | Decrease               |
| ALC9200           | G      | 64                 | 128                    | 12 (R)           | No decrease          | No decrease            |
| ALC9196           | G      | 64                 | 64                     | 2 (I)            | No decrease          | No decrease            |
| C36 parent        | G      | 512                | 1024                   | 12 (R)           | No decrease          | No decrease            |
| ALC9259           | G      | 64                 | 64                     | 2 (I)            | No decrease          | No decrease            |
| ALC9268           | T      | 64                 | 64                     | 1.5 (S)          | No decrease          | No decrease            |
| ALC9322           | T      | 64                 | 64                     | 0.25 (S)         | No decrease          | No decrease            |

*CA-MHB, cation-adjusted Mueller Hinton Broth.

1Susceptible (S) PEN + CLAV MIC is an MIC of < 2 µg/mL by Etest on ISA media; intermediate (I) PEN + CLAV MIC is an MIC = 2 µg/mL by Etest on ISA media; resistant (R) PEN + CLAV MIC is an MIC > 2 µg/mL by Etest on ISA media.

2Exposure to 44 mM NaHCO₃ 1/2 × MIC oxacillin results in decreased, increased, or no change in meCA expression compared to exposure to 1/2 × MIC oxacillin alone by qRT-PCR.

3Exposure to 44 mM NaHCO₃ 1/2 × MIC oxacillin results in decreased PBP2a protein production compared to exposure to 1/2 × MIC oxacillin alone by Western blotting.
from T-to-G eliminated the NaHCO₃-responsiveness phenotype to OXA in this background, such that mutants harboring the RBS -7G allele (ALC9200 and ALC9196) were highly resistant to OXA (Table 1). Despite the ability of the RBS -7G allele to reverse the NaHCO₃-responsive phenotype to OXA in the MW2 background, introduction of the RBS -7T allele into the C36 nonresponsive parental background (ALC9268 and ALC9322) did not establish the responsive phenotype to OXA (Table 1). The ability of the RBS site mutation to alter penicillin/clavulanic acid susceptibility, but not NaHCO₃-mediated β-lactam susceptibility in the nonresponsive strain background, indicates that additional elements are involved in the NaHCO₃-responsive phenotype besides these specific mecA RBS genotypes.

**Effect of mecA alleles on mecA transcription, translation, and PBP2a production/localization.** To assess the impact of alterations to the RBS/promoter region of mecA on its transcription, mecA gene expression after OXA induction was quantified by qRT-PCR, in the presence and absence of NaHCO₃ for MW2 and C36 parental strains, as well as their respective mecA variant swap constructs. Although the mecA start codon upstream -7 site has been identified as part of the putative ribosomal-binding site (RBS), point mutations at this location have been reported to impact on mecA transcription (27, 28). We previously demonstrated that NaHCO₃ repressed expression of mecA specifically in NaHCO₃-responsive MRSA strains, but not in nonresponsive strains (10, 20). When mecA transcription was assessed in the absence of NaHCO₃, similar expression levels were observed for both parental strains and their swap constructs (Fig. 1A and B). However, in the presence of NaHCO₃, mecA transcription was highly repressed compared to expression in the absence of NaHCO₃ for MW2 parent and mutants harboring the RBS -7T allele; in contrast, mecA expression was not repressible by NaHCO₃ for MW2 swap mutants harboring the RBS -7G allele (Fig. 1A). In the C36 strain background,
introduction of the RBS -7T allele did not result in NaHCO3-mediated repression of mecA (Fig. 1B).

To determine the influence of altering the RBS -7 site on mecA translation in MW2 and C36 strain backgrounds, translational GFP reporter fusion constructs were generated for the native MW2 and C36 mecA promoter regions, and swap-transformed back into MW2 and C36. Translational efficiency of each GFP fusion construct was assessed by flow cytometry, in the presence and absence of NaHCO3. Interestingly, unlike the transcriptional data, alteration of the RBS -7 site had substantial impact on the translational efficiency of each construct, regardless of the strain background. In both MW2 and C36, strains harboring the RBS -7G construct (C36 native mecA promoter sequence) had significantly more GFP production than those harboring the RBS -7T construct (MW2 native mecA promoter sequence) (Fig. 2). Of note, growth in NaHCO3-containing media did not reduce the translational efficiency of the RBS -7T fusion, and slightly enhanced translation of the RBS -7G fusion in the C36 background (Fig. 2). Taken together with the mecA expression data, this indicates that NaHCO3 may be specifically impacting mecA expression more preferentially at the transcriptional, rather than at the translational level, in responsive strains.

Finally, to determine the overall impact of specific mecA alleles on PBP2a production and membrane localization, Western blotting was performed on the membrane protein fraction of MW2 and C36 mecA mutant constructs grown in the presence and absence of NaHCO3. In the MW2 background, strains harboring the RBS -7T variant had reduced amounts of membrane-localized PBP2a when grown in the presence versus absence of NaHCO3 (Fig. 3A), corresponding to the observed repression of mecA transcription in the presence of NaHCO3 observed in these strains (Fig. 1A). MW2 strains harboring the RBS -7G allele had overall increased membrane-associated PBP2a in both the presence and absence of NaHCO3 compared to strains harboring the RBS -7T allele (Fig. 3A); this outcome corresponded to the increased translational efficiency of this sequence variant (Fig. 2). In the C36 background, all strains had similar or increased levels of membrane PBP2a in the presence versus absence of NaHCO3 (Fig. 3B), and no consistent pattern was seen between the RBS -7T and -7G variant.

DISCUSSION

The recent discoveries of the impact of specific mecA alleles on β-lactam susceptibility phenotypes appear to reveal novel paradigms for further understanding of β-lactam resistance in MRSA. Thus, mutations within the mecA promoter at the -7 site (a part of the RBS) have now been linked to both altered susceptibility to a combination of β-lactam/β-lactamase inhibitors, as well as to the NaHCO3 β-lactam-responsive phenotype (27, 30).

A major change following the swap-in at the RBS -7 site into the NaHCO3-responsive...
strain, MW2, appeared to be at the mecA transcriptional level. Previously, NaHCO₃ was shown to repress expression of mecA specifically in NaHCO₃-responsive strains, such as MW2 (10, 20). Herein, we observed that MW2 harboring the native RBS -7T allele had the predicted NaHCO₃-repressible mecA expression outcome; in contrast, mecA expression in swap mutants harboring the RBS -7G allele was either not repressed or enhanced by NaHCO₃. Interestingly, mecA expression in the absence of NaHCO₃ was similar for all mutant strains within a given strain background (MW2 and C36). This finding is contrary to that identified by Harrison et al. (27) and Chen et al. (28), whereby alteration of the RBS -7 site from T-to-G resulted in enhanced mecA transcription under OXA-induction conditions (27). However, it should also be underscored that Harrison et al. and others reported individual strain-to-strain variations in the latter relationship (27, 29); this seems to indicate that other strain-specific factors or environmental cues (e.g., in growth media) influence how mecA promoter mutations affect mecA transcription. Lastly, in our studies, alteration of the RBS -7 from T-to-G did result in a substantial increase in PBP2a translation in both strain backgrounds, as well as PBP2a membrane protein production in the MW2 background (and in one of the two swaps in C36); these latter data are similar to a previous report (28), and more consistent with this site’s identified role within the RBS.

Based on these current data-sets, one likely mechanism involved in reversing NaHCO₃ responsiveness in MW2 swap strains may be a de-repression of mecA expression by NaHCO₃ in swap strains harboring the RBS -7G allele. Such an event would result in increased PBP2a production and higher OXA MICs in the presence of NaHCO₃. Interestingly, other studies have found that alteration of mecA/PBP2a expression does not necessarily directly correlate to β-lactam MIC levels (28, 31–33). However, our data reveal a more direct correlation between PBP2a protein levels and MICs for all strains and conditions observed in this study. This was particularly noted for the C36 parental strain, which produced substantially more PBP2a than the other three C36 mecA swap variant strains; their MICs in media with and without NaHCO₃ were 2- to 8-fold greater than the other mecA variants within this background. These data indicate that strain-specific factors influence PBP2a production, dictating the specific MIC for that strain. Of note, alteration of the RBS -7 G-to-T sequence in the C36 background did not result in NaHCO₃-mediated repression of mecA/PBP2a. Although this observation explains why this genetic alteration did not evoke a NaHCO₃-responsiveness phenotype in this strain background, it raises the notion that other factors, besides the mecA RBS sequence, must be required to stimulate NaHCO₃-mediated mecA gene repression to subsequently yield a NaHCO₃-responsive phenotype.

Taken together, these data support a primary role of the RBS -7 site as a mediator of mecA transcription and PBP2a translation in the NaHCO₃-responsive strain, MW2. Although the RBS -7 site is clearly important for the maintenance of the responsive phenotype, it does not appear to be sufficient to generate this phenotype in a nonresponsive strain.
background. More work must be undertaken in additional MRSA strain backgrounds to elu-
cidate the genetic and molecular mechanisms required to generate the NaHCO₃-responsive phenotype, and to understand their role in the context of mecA transcriptional regulation.

One possible explanation for the differential ability of NaHCO₃ to repress mecA trans-
scription in the MW2 versus C36 background strains may be intrinsic differences in the
native SCCmec cassettes in each strain background. NaHCO₃-responsive strain, MW2, pos-
sesses SCCmec type IV, whereas the nonresponsive strain C36 possesses SCCmec type II
(30). Although linkage between specific SCCmec types and the NaHCO₃-responsive versus-
nonresponsive phenotypes has not been established (24), the underlying genetic differences
between these two cassettes may contribute to the ability of specific mecA genotypes to
counter NaHCO₃-responsiveness. Specifically, the SCCmec type IV cassette has truncations
within mecI-mecR1, encoding the mecA inhibitor gene (mecI) and the cognate response reg-
ulator (mecR1), rendering these genes nonfunctional; in contrast, the SCCmec type II cassette
has an intact and functional mecA-mec-mecR1 regulatory axis (34–36). The lack of an intact
mecI-mecR1 system in the responsive strain, MW2, indicates mecA expression is probably
regulated by β-lactamase-regulatory elements, blal-blalR1, and possibly other, as yet unde-
defined regulatory systems. Alternatively, mecA expression in strains within the C36 genetic
background may be more tightly regulated by their intact mecI-mecR1 system. It might be
speculated that in the presence of functional mecI-mecR1, alteration of the RBS -7G-to-T is
insufficient to allow for NaHCO₃-mediated repression of mecA transcription. Conversely, in
the absence of intact mecI-mecR1, the RBS -7T allele can confer NaHCO₃-mediated repression
of mecA. Further studies are being carried out to explore these possibilities.

Finally, it should be noted that some of the disparity between the ‘swap’ results, in
terms of penicillin-clavulanate versus OXA-NaHCO₃-responsiveness MIC metrics may have
been influenced by the differential PBP2a binding affinity of penicillin versus OXA, which
differ by ~20-fold (18).

Of note, we did not observe any impacts of alteration of the 246th amino acid position
alone on the NaHCO₃-responsive phenotype in either strain background, despite this mutation
being associated with altered susceptibility to β-lactam/β-lactamase inhibitor combi-
nations (27). Interestingly, in a separate study, we did observe that purified PBP2a 246E
and 246G protein variants had differential binding affinities for Bocillin-FL in the presence of
NaHCO₃ (37). These latter data imply that the specific PBP2a protein variant present in a
strain background may alter the binding affinity of a given strain for β-lactams in the pres-
ence of NaHCO₃, although on its own, this polymorphism is not sufficient to alter the
NaHCO₃-responsive phenotype of a given strain. We hypothesized that this difference in
β-lactam binding affinity between the two PBP2a variants in the presence of NaHCO₃ may
be due to an altered NaHCO₃ buffering capacity between the glutamic acid (246E) and glycine
(246G) residues present in their specific allosteric binding domains (22).

Overall, this study elucidates the impact of mecA sequence polymorphisms on the
NaHCO₃-responsive phenotype in MRSA, and sheds further light on the complex regulation
of methicillin resistance in S. aureus.

MATERIALS AND METHODS

Strains, media, and growth conditions. The primary parental strains utilized in this study were the
prototypical and well-characterized NaHCO₃-responsive strain, MW2 (10, 20, 30, 38) and the nonrespon-
sive strain, C36 (15, 30) (Table 2). Strains MW2 and C36 have previously been identified as having either the
“susceptible 2” or “resistant 2” mecA genotypes, respectively (30), as defined by Harrison et al. (27). Strains
were stored at −80°C and isolated on tryptic soy agar (TSA) at 37°C in ambient air when ready for use. All liq-
uid cultures were grown at 37°C in ambient air with aeration.

For penicillin Etest susceptibility testing of penicillin-clavulanate combinations, Iso-Sensitest Agar
(IS, Oxoid) was prepared as per manufacturer’s instructions, with or without 15 μg/mL clavulanic acid (Sigma-Aldrich; see below for further details). For broth MIC testing, RNA isolation/gene expression studies,
GFP reporter assays, and Western blotting, strains were cultured in cation-adjusted Mueller-Hinton Broth
(CA-MHB, Difco, Beckton-Dickinson) or CA-MHB buffered with 100 mM Tris (CA-MHB Tris) to maintain pH
7.3 ± 0.1, with or without 44 mM NaHCO₃. Where indicated, 1/2 × MIC of OXA (Table 1) was also added to
the growth media to stimulate mecA expression. In all experiments in which OXA was added to the growth medium, 2% NaCl was also included.

Construction of various S. aureus mutant and reporter strains. To determine the contribution of the
-7-nucleotide position (AGGAGG/T) (corresponding to the ribosome-binding site, RBS [28]) from the
ATG start codon or the amino acid position at 246-residue (Glu/Gly) of the mecA gene in NaHCO₃-responsive or nonresponsive S. aureus strains, we have constructed chromosomal point mutations of the mecA region in S. aureus strains MW2 and C36 (erm³) using routine procedures as described (39). To construct mutations or interchange the region, a 3.2 kb DNA fragment was amplified that contained the intact mecA and mecR genes by PCR using primers flanking with BamHI site at both ends (Table S1). The DNA fragment was cloned into a temperature-sensitive shuttle vector pMAD (β-gal, erm³) (40) or pMAD-X (β-gal, modiﬁed with chlorR) by removing erm³, and then selected in E. coli IM08B (41) for the correct construct. To construct point mutations at the RBS or at the 246th residue position, site-specific mutagenesis was performed with pMAD constructs as the template and various mutagenized primers using a PCR based method with Pfu/Taq-polymerase (Phusion, Thermo Scientiﬁc). After veriﬁcation by restriction digestion and DNA sequencing, interchanged or point mutation constructs were introduced into various strains by electroporation and selected on erythromycin or chloramphenicol and X-Gal-containing plates for blue colonies at 30°C. Plasmid DNA was isolated and digested with BamHI for the authentication of the presence of DNA fragment in the respective constructs in the strains. The construction of chromosomal mutations in the respective strain by recombination or two-point crossover was performed by routine procedure as described previously (39). Briefly, two-point crossover of the mecA-mecR region was performed by temperature shift by growing at strains 33°C with erythromycin or chloramphenicol followed by 30°C subculturing without any antibiotics. Cells were plated with and without erythromycin or chloramphenicol in the presence of X-Gal (40 μg/mL) for selection and incubated at 37°C. White/non-blue colonies were cross-streaked to select erm³ (MW2) or chlorR (C36) colonies for the potential two-point crossover clones or mutants. The mutants were veriﬁed by chromosomal PCR and DNA sequencing of the PCR product.

To determine if AGGAGG in C36 (nonresponsive) or AGGAGT in MW2 (NaHCO₃-responsive) RBS sequence variations have any role in the NaHCO₃-responsiveness phenotype, translational fusions were constructed for these two-promoter regions. The 95-bp intergenic promoter region of the mecA-mecR genes was cloned into a promoter-less gfpuvr reporter shuttle plasmid, pALC1484, at EcoRI and XbaI sites (Fig. S1) (42). Final constructs were veriﬁed by DNA sequencing and mobilized into MW2 and C36 strains.

**Etest and broth microdilution susceptibility testing.** E-testing was performed on iso-sensitest agar (ISA) with or without 15 μg/mL clavulanic acid as previously described (27). Briefly, cells were grown overnight in 1 mL of tryptic soy broth (TSB), then diluted to 1 × 10⁸ CFU/mL in phosphate-buffered saline (PBS) and plated via the Kirby-Bauer inoculation method (43). Etest strips containing benzylpenicillin (bioMérieux) were placed on the inoculated plates, and plates were incubated overnight at 37°C in ambient air. Broth microdilution MICs were performed according to CLSI guidelines as previously described (10, 44, 45). Briefly, cells were grown overnight in the indicated media condition (CA-MHB, CA-MHB Tris, or CA-MHB Tris + 44 mM NaHCO₃), then diluted into the same media, containing 2% NaCl, with 2-fold serial dilutions of OXA (Sigma-Aldrich) at a ﬁnal cell concentration of 5 × 10⁸ CFU/mL. Plates were incubated overnight at 37°C in ambient air and the MIC was read as the ﬁrst well in which visual turbidity was reduced compared to the no drug control well.

**RNA isolation and qRT-PCR analysis of mecA gene expression.** RNA was isolated from stationary-phase cells grown in CA-MHB Tris ± 44 mM NaHCO₃, with 1/2× MIC OXA and 2% NaCl as previously described (10). RNA was released from cell pellets by FastPrep disruption (FP120, Thermo Savant) in Lysing Matrix B tubes (MP Biomedicals) and isolated by column puriﬁcation (Qiagen). RNA samples were subjected to Turbo DNase treatment (Turbo DNA-free, Invitrogen, Thermo Fisher Scientiﬁc) and reverse transcribed to generate a cDNA library (Superscript IV, Invitrogen, Thermo Fisher Scientiﬁc). The mecA gene transcript was detected by qPCR (StepOne, Applied Biosystems) using primers listed in Table S1. gyrB was used as a housekeeping gene to

### TABLE 2 Strains and plasmids used in this study

| Strains or plasmids | Relevant features | Reference |
|---------------------|------------------|-----------|
| MW2 (parent)        | mecA genotype: RBS -7T, 246G; CC type: 1; spa type: t128; SCCmec type: IV | (30, 38) |
| ALC9188 (MW2 derivative) | mecA genotype: RBS -7T, 246E | This study |
| ALC9200 (MW2 derivative) | mecA genotype: RBS -7G, 246G | This study |
| ALC9196 (MW2 derivative) | mecA genotype: RBS -7G, 246E | This study |
| C36 (parent)        | mecA genotype: RBS -7G, 246G; CC type: 5; spa type: t002; SCCmec type: II | (15, 30) |
| ALC9259 (C36 derivative) | mecA genotype: RBS -7G, 246G erm³ | This study |
| ALC9268 (C36 derivative) | mecA genotype: RBS -7T, 246G erm³ | This study |
| ALC9322 (C36 derivative) | mecA genotype: RBS -7T, 246G erm³ | This study |
| Plasmids            |                  |           |
| ALC9332 (MW2 derivative) | pALC1484 pC36mecA:gfpuvr chlorR | This study |
| ALC9334 (MW2 derivative) | pALC1484 pMW2mecA:gfpuvr chlorR | This study |
| ALC9329 (C36 derivative) | pALC1484 pC36mecA:gfpuvr chlorR | This study |
| ALC9330 (C36 derivative) | pALC1484 pMW2mecA:gfpuvr chlorR | This study |
| pMAD                 | β-gal, erm³ | (40) |
| pMAD-X               | β-gal, chlorR, modified pMAD with cat gene by removing erm gene | This study |
| pALC1484             | Promoter-less gfpuvr chlorR | (42) |
normalize transcript quantities. Relative gene expression was calculated by the ΔΔCt method on biological replicates performed in triplicate in at least two independent runs.

Assessment of mecA promoter-GFP fusions by flow cytometry. To determine the activity of native MW2 and C36 promoters, GFP production in the reporter strain constructs was measured by flow cytometry. Reporter strains containing the pALC1484 plasmid (Table 2), were grown overnight in CA-MHB Tris ± 44 mM NaHCO₃, with 1/2× MIC oxacillin and 2% NaCl, pelleted, and incubated with DNase (Ambion, Invitrogen), RNase (Thermo Fisher Scientific), and Halt Protease Inhibitor Cocktail (Thermo Fisher Scientific) for 30 min at 37°C and then 15 min at 4°C as previously described (20). The cells were disrupted with glass beads by FastPrep agitation (FP120, Thermo Savant), and centrifuged for 10 min at 4°C and 15,000 RPM to clarify the suspension. The membrane protein fraction was collected from the supernatant by centrifugation for 2h at 4°C and 15,000 RPM and resuspended in PBS containing Halt Protease Inhibitor. Membrane protein concentration was quantified by Bradford protein assay; 40 μg of membrane proteins were separated on a 4–12% Bis-Tris gel (Invitrogen), run with MES buffer, and blotted onto a nitrocellulose membrane (Amersham). Total protein loading was confirmed by staining with 0.25% ponceau (Fig. S2). The membrane was blocked with 10% dry milk in Tris Buffered Saline with Tween (TBST). PBP2a was probed with a chicken anti-PBP2a antibody (RayBiotech) diluted 1:2500 and detected with an anti-chicken IgG cross-absorbed secondary antibody, HRP (Thermo Fisher Scientific) diluted 1:5000. Labeled proteins were imaged using a c400 imager (Azure Biosystems).

SUPPLEMENTAL MATERIAL
Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.3 MB.

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