Impact of Bicarbonate on PBP2a Production, Maturation, and Functionality in Methicillin-Resistant *Staphylococcus aureus*

Selvi C. Ersoy,[a] Henry F. Chambers,[b] Richard A. Proctor,[c] Adriana E. Rosato,[d] Nagendra N. Mishra,[a,e] Yan Q. Xiong,[a,e] Arnold S. Bayer[a,e]

[a]The Lundquist Institute, Torrance, California, USA
[b]UCSF School of Medicine, San Francisco, California, USA
[c]Departments of Medicine and Medical Microbiology/Immunology, University of Wisconsin School of Medicine and Public Health, Madison, Wisconsin, USA
[d]Department of Pathology, Riverside University Health Systems, Riverside, California, USA
[e]Geffen School of Medicine at UCLA, Los Angeles, California, USA

ABSTRACT Certain methicillin-resistant *Staphylococcus aureus* (MRSA) strains exhibit β-lactam susceptibility *in vitro*, *ex vivo*, and *in vivo* in the presence of $NaHCO_3$ ($NaHCO_3$-responsive MRSA). Here, we investigate the impact of $NaHCO_3$ on factors required for PBP2a functionality. Prototype $NaHCO_3$-responsive and -nonresponsive MRSA strains (as defined *in vitro*) were assessed for the impact of $NaHCO_3$ on the expression of genes involved in PBP2a production-maturation pathways (*mecA*, *blaZ*, *pbp4*, *vraSR*, *prsA*, *sigB*, and *floA*), membrane PBP2a and PrsA protein content, and membrane carotenoid content. Following $NaHCO_3$ exposure in $NaHCO_3$-responsive (versus nonresponsive) MRSA, there was significantly reduced expression of (i) *mecA* and *blaZ*, (ii) the *vraSR*-*prsA* gene axis, and (iii) *pbp4*. Carotenoid production was reduced while *floA* expression was increased by $NaHCO_3$ exposure in all MRSA strains. This work underscores the distinct regulatory impact of $NaHCO_3$ on a cadre of genes encoding factors required for the maintenance of the MRSA phenotype through PBP2a functionality and maturation.

KEYWORDS methicillin-resistant *Staphylococcus aureus* (MRSA), sodium bicarbonate ($NaHCO_3$), beta-lactam, penicillin-binding proteins (PBP), PBP2a

*S*taphylococcus aureus* is the major causative agent of a number of serious clinical syndromes and a notable public health threat (1–3). Methicillin-resistant *S. aureus* (MRSA) has been a particular problem due, in part, to its assumed recalcitrance to many first-line therapies useful in the treatment of methicillin-susceptible *S. aureus* (MSSA) strains, especially β-lactam agents (4). Current anti-MRSA therapies tend to be costlier, less effective, and/or more toxic than those used to treat MSSA infections (e.g., daptomycin, linezolid, and vancomycin [5, 6]); also, patients with MRSA infections are prone to prolonged hospital stays, imposing large economic burdens for their treatment (7).

MRSA strains are preemptively identified in most clinical microbiology laboratories by phenotypic assays (e.g., cefoxitin disk diffusion or PBP2a latex agglutination) (8), as well as by the presence of the *mecA* gene cassette, encoding the alternative penicillin-binding protein 2a (PBP2a). Additionally, minimum inhibitory concentration (MIC) determinations, as outlined by the Clinical and Laboratory Standards Institute (CLSI), are used to confirm their resistance to standard β-lactam antibiotics (i.e., oxacillin) (9, 10). The CLSI considers *S. aureus* isolates whose MICs to oxacillin are ≥ 4 μg/ml in cation-adjusted Mueller-Hinton broth (CA-MHB) to be oxacillin resistant (and resistant to first-generation cephalosporins) (4, 9); the treatment of such strains with all β-lactam agents (excluding ceftaroline and ceftobiprole) is discouraged by published guidelines (4, 9).

We recently discovered that the addition of $NaHCO_3$, the body's primary biological

Address correspondence to Arnold S. Bayer, abayer@lundquist.org.

buffer, to CA-MHB rendered a relatively large proportion of MRSA strains susceptible to two standard β-lactams, oxacillin and cefazolin, by MIC testing; this phenotype has been termed NaHCO$_3$ responsiveness (11, 12). The translational relevance of this NaHCO$_3$-responsive phenotype was verified in a small strain set by successful β-lactam therapy in an *ex vivo* simulated endocarditis vegetation model as well as in a rabbit model of infective endocarditis (11, 13).

Prior studies suggested that NaHCO$_3$ exerted its impact on antimicrobial susceptibility by targeting and collapsing the proton motive force (PMF) (14); however, this would not adequately explain β-lactam resensitization by NaHCO$_3$. Instead, we recently determined that coexposure of these *in vitro*-responsive MRSA strains to NaHCO$_3$ plus oxacillin reduced the expression of both *mecA* and *sarA*, associated with reduced PBP2a protein production (11). The latter observations were important, as oxacillin binds relatively poorly to PBP2a (encoded by *mecA*), and deletion of *sarA* can increase susceptibility to oxacillin, in part, by influencing *mecA* expression (15).

The above-described finding of NaHCO$_3$-mediated reduction of *sarA* expression suggested an additional mechanism by which NaHCO$_3$ could influence β-lactam sensitization in MRSA, i.e., via an impact on cell membrane (CM) physiology and PBP2a maturation. The expression of *sarA* is regulated by the alternative sigma factor, SigB (16), which, in turn, controls the staphyloxanthin biosynthesis operon, *crtOPQMN* (17). The staphyloxanthin carotenoid pigment, along with flotillin (encoded by *floA*), are integral parts of functional membrane microdomains (FMMs), which, importantly, provide a scaffolding that anchors PBP2/2a proteins within the MRSA CM (18, 19).

Defining the potential impacts of NaHCO$_3$ on other key aspects of PBP2a regulation (e.g., *blaZ* expression), maturation (e.g., *vraSR* and *prsA* expression), and peptidoglycan cross-linking (e.g., *pbp4*) is critical to developing a more complete understanding of bicarbonate's effect on oxacillin susceptibility. Thus, genes within the *blaZ* operon coregulate *mecA* expression (20). Of note, VraSR and PrsA have been identified as critical to the see-saw effect, in which daptomycin-nonsusceptible MRSA strains exhibit β-lactam hypersusceptibility (21–23). VraSR is a two-component regulatory system that positively regulates the expression of PrsA, a chaperone required for proper PBP2a maturation, localization, and functionality (24, 25). Moreover, PBP4 is a PBP with transpeptidase activity required to complete peptidoglycan cross-linking in the presence of oxacillin (26, 27).

The current study was designed to adjudicate the above-described preliminary findings in a larger collection of NaHCO$_3$-responsive and -nonresponsive MRSA strains as well as to further define key genetic determinants of these two distinct microbiologic phenotypes.

(These data were presented, in part, at the 4th Annual Texas Medical Center Antimicrobial Resistance and Stewardship Conference [28].)

RESULTS

Impact of NaHCO$_3$ on *mecA* and *blaZ* expression. The *blaZ* operon is important in coregulating *mecA* expression and PBP2a production (20). As shown in Fig. 1, NaHCO$_3$ exposure substantially decreased *mecA* gene expression under oxacillin-inducing conditions in all four NaHCO$_3$-responsive strains. In contrast, *mecA* expression was increased during NaHCO$_3$ exposure in the four nonresponsive strains (Fig. 1A). Similarly, *blaZ* expression was significantly reduced in all four NaHCO$_3$-responsive strains while being significantly increased in two of the three β-lactamase-positive, NaHCO$_3$-nonresponsive strains (COL is naturally β-lactamase negative) (Fig. 1B).

Influence of NaHCO$_3$ on PBP2a content. As previously observed in a smaller set of MRSA strains (11), growth in media containing NaHCO$_3$ reduced the amount of PBP2a in NaHCO$_3$-responsive versus nonresponsive strains (Table 1; see also Fig. S2 in the supplemental material).

To further verify the impact of NaHCO$_3$ on PBP2a protein production and its CM localization, Western blotting was performed on CM protein extracts from cells grown in the presence of 0.5× MIC of oxacillin (to induce *mecA* expression), with or without NaHCO$_3$ (Fig. 2). As predicted by the known NaHCO$_3$ repression of the coregulatory
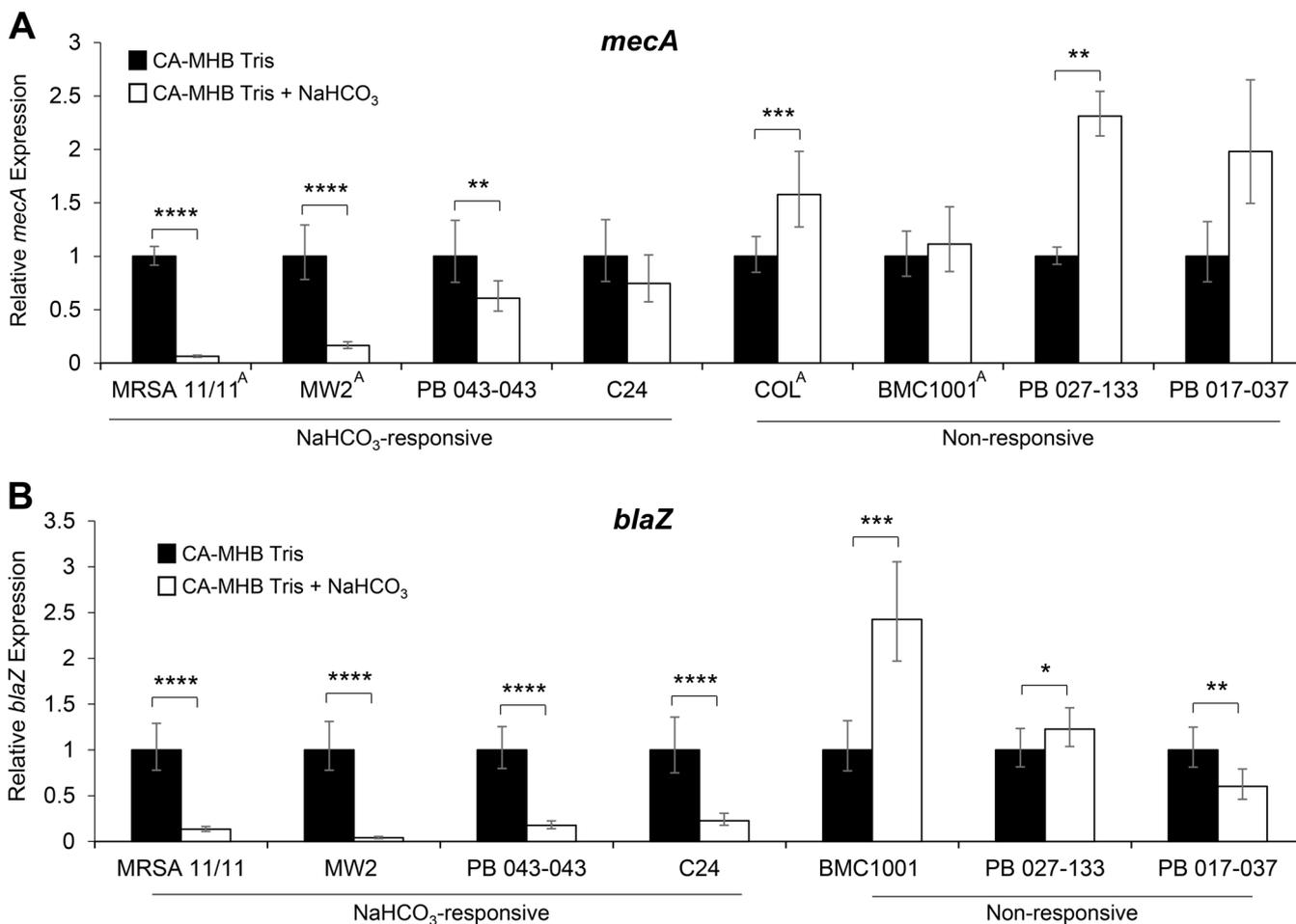
FIG 1 Expression of *mecA* (A) and *blaZ* (B) in the presence and absence of NaHCO₃. qRT-PCR was performed using RNA extracted from stationary-phase cells in the presence of $0.5 \times$ MIC of oxacillin. Oxacillin concentrations used are the following: MRSA 11/11, without NaHCO₃ (w/o), 16 µg/ml; with NaHCO₃ (+ NaHCO₃), 0.25 µg/ml; MW2, w/o, 32 µg/ml; + NaHCO₃, 1 µg/ml; PB 043-043, w/o, 8 µg/ml; + NaHCO₃, 0.5 µg/ml; C24, w/o, 16 µg/ml; + NaHCO₃, 0.5 µg/ ml; COL, w/o, 256 µg/ml; + NaHCO₃, 256 µg/ml; BMC1001, w/o, 128 µg/ml; + NaHCO₃, 128 µg/ml; PB 027-133, w/o, 128 µg/ml; + NaHCO₃, 128 µg/ml; PB 017-037, w/o, 16 µg/ml; + NaHCO₃, 16 µg/ml. Black bars indicate growth in CA-MHB 100 mM Tris, white bars indicate growth in CA-MHB 100 mM Tris plus 44 mM NaHCO₃, and error bars indicate the C_T standard deviations. No *blaZ* expression was detected in COL, because the strain lacks the *blaZ* gene. Student's *t* test was used for statistical analyses. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. *mecA* gene expression data for strains marked with a superscript A were previously published [11].

mecA-blaZ gene axis expression in NaHCO₃-responsive strains, CM-specific PBP2a protein production was visibly decreased in the presence of NaHCO₃ in three of four NaHCO₃-responsive strains (Fig. 2A). In contrast, NaHCO₃ exposure had no impact on PBP2a production in any of the NaHCO₃-nonresponsive strains (Fig. 2B). These data indicated that not only was total PBP2a production downregulated in NaHCO₃-responsive strains (as per the agglutination assay) but also the amount of protein specifically inserted into the CM was similarly impacted.

Effect of NaHCO₃ on genes involved in PBP2a maturation and localization. For optimal PBP2a protein functionality, the PrsA chaperone must facilitate posttranslational maturation of PBP2a (21, 25). Regulation of *prsA* gene expression typically occurs via VraSR, a two-component regulatory system known to be involved in the daptomycin resistance/oxacillin hypersusceptibility see-saw effect (21, 23). Interestingly, NaHCO₃ highly repressed expression of both *vraSR* and *prsA* only in NaHCO₃-responsive strains (Fig. 3A and B).

Furthermore, as predicted by the quantitative real-time PCR (qRT-PCR) gene expression data described above, less PrsA protein was present in the CMs of all NaHCO₃-responsive MRSA following NaHCO₃ exposure versus cells grown in the absence of NaHCO₃ (Fig. 4A). In contrast, NaHCO₃ had no apparent effect on PrsA CM content in

TABLE 1 PBP2a agglutination of NaHCO$_3$-responsive and nonresponsive strains grown in medium with or without 44 mM NaHCO$_3$

	PBP2a agglutination level[a]	
Strain	CA-MHB Tris	CA-MHB Tris 44 mM NaHCO$_3$
MRSA 11/11[b]	++	−
MW2[b]	++	−
PB 043-043	+++	+
C24	+++	+
COL[b]	+++	+++
BMC1001[b]	+++	+++
PB 027-133	+++	+++
PB 017-037	+++	+++

[a]Interpretation of agglutination intensity: +++, high; ++, moderate; +, low; −, none.
[b]Data were previously reported (11).

three NaHCO$_3$-nonresponsive strains (Fig. 4B). NaHCO$_3$ did not consistently impact extracellular secretion patterns of PrsA in either NaHCO$_3$-responsive or -nonresponsive MRSA (data not shown). These data support the notion that in the presence of oxacillin induction, NaHCO$_3$ reduces overall PrsA production in NaHCO$_3$-responsive MRSA, resulting in decreased PrsA CM content (as opposed to excess PrsA secretion extracellularly, as seen in the see-saw effect [21]).

***pbp4* expression.** In addition to proper protein folding, the PBP2a protein requires PBP4 activity to complete cell wall synthesis (26). Although PBP2a provides the transpeptidase activity involved in peptidoglycan synthesis, highly cross-linked peptidoglycan cannot be produced in many MRSA strains in the presence of oxacillin without PBP4 (18, 26, 29). The qRT-PCR analysis revealed that *pbp4* expression was substantially and selectively repressed in the presence of NaHCO$_3$ in NaHCO$_3$-responsive (but not in NaHCO$_3$-nonresponsive) strains (Fig. 3C); this suggested that a less highly cross-linked peptidoglycan is produced in NaHCO$_3$-responsive strains in the presence of NaHCO$_3$.

Influence of NaHCO$_3$ on factors impacting FMM scaffolding for PBP2a. The alternative sigma factor and stress response regulator, SigB, is involved in regulating genes involved in CM biophysics (e.g., fluidity/rigidity), notably the staphyloxanthin carotenoid biosynthesis operon, *crtOPQMN* (17). In turn, carotenoids and flotillin (FloA) are critical in providing a key scaffolding upon which PBP2a can oligomerize (19). Additionally, the global regulon *sarA* is also SigB regulated and is important in maintaining the MRSA phenotype (15, 16, 30). Further, as noted above, we previously determined that NaHCO$_3$ was capable of repressing *sarA* expression in NaHCO$_3$-responsive strains (11). Thus, we hypothesized that NaHCO$_3$ influences SigB activity, resulting in downstream impacts on SigB-regulated phenotypes important in PBP2a functionality.

The expression of the constitutively active *sigB* gene was assessed through expres-
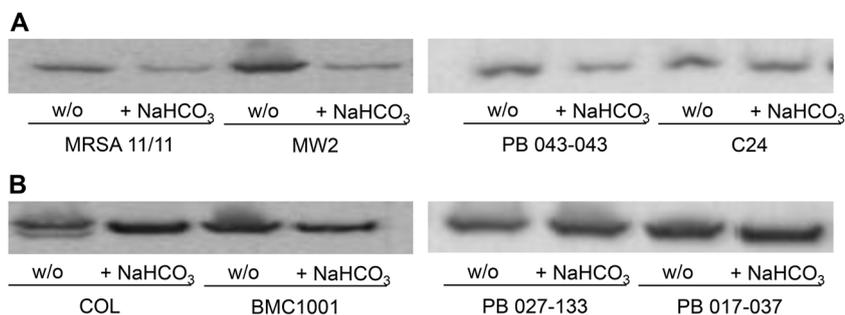
FIG 2 Membrane PBP2a protein content in MRSA strains grown in the presence and absence of NaHCO$_3$. (A) NaHCO$_3$-responsive strains. (B) NaHCO$_3$-nonresponsive strains. Strains were grown in CA-MHB plus 100 mM Tris (w/o) or CA-MHB plus 100 mM Tris plus 44 mM NaHCO$_3$ (+ NaHCO$_3$) in the presence of 0.5× MIC oxacillin. Proteins are the cell membrane fraction extracted from stationary-phase cells.
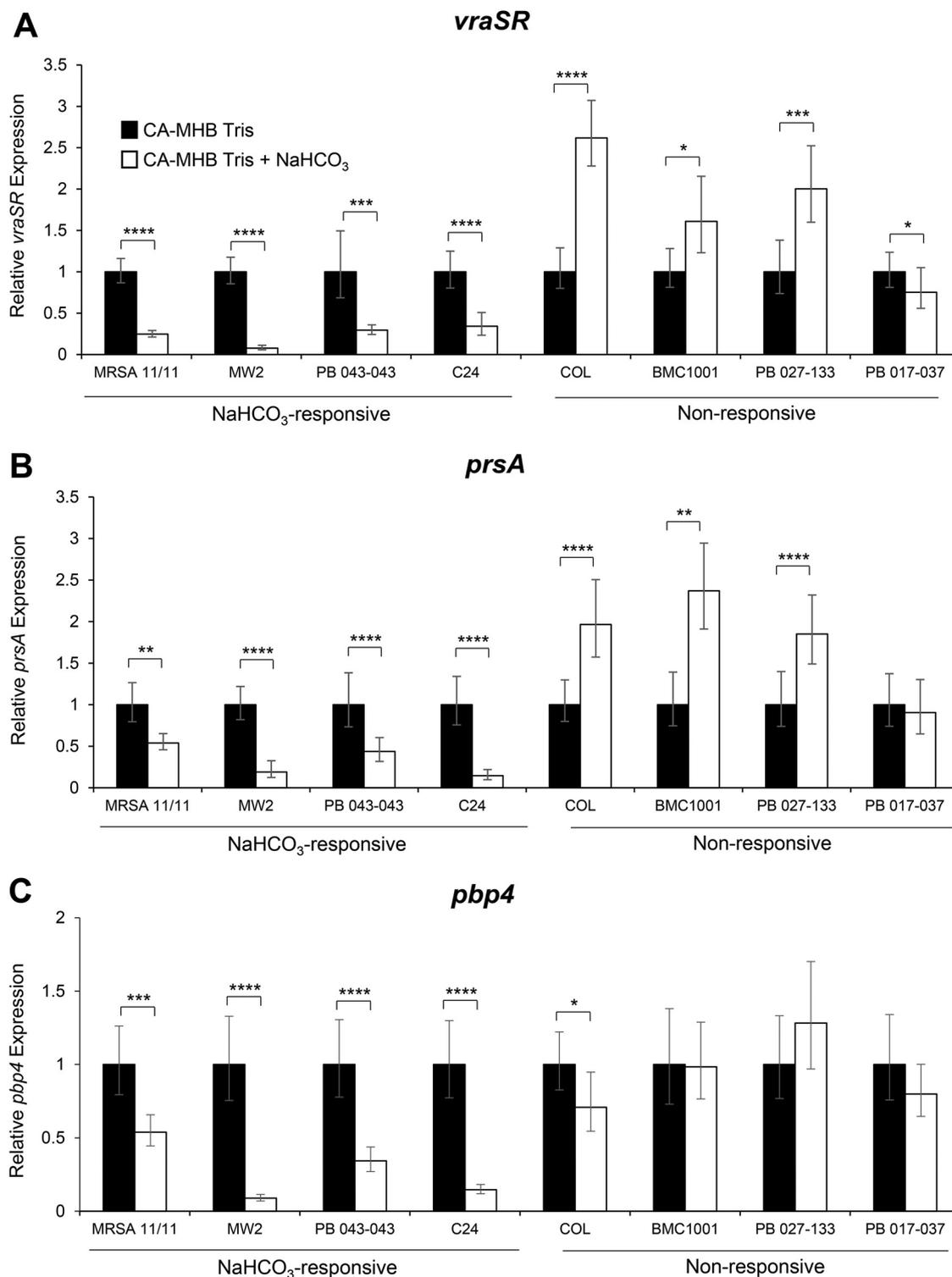
FIG 3 Expression of genes related to PBP2a maturation and function in the presence and absence of NaHCO$_3$. (A) vraSR. (B) prsA. (C) pbp4. qRT-PCR was performed using RNA extracted from log-phase cells (OD$_{600}$, 0.4) in the presence of 0.5× MIC oxacillin for vraSR and prsA or on RNA extracted from stationary-phase cells (pbp4). Black bars indicate growth in CA-MHB with 100 mM Tris, white bars indicate growth in CA-MHB with 100 mM Tris plus 44 mM NaHCO$_3$ for all graphs, and error bars indicate the C$_T$ standard deviations. Student's t test was used for statistical analyses. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

A

w/o	+ NaHCO$_3$	w/o	+ NaHCO$_3$	w/o	+ NaHCO$_3$	w/o	+ NaHCO$_3$
MRSA 11/11		MW2		C24		PB 043-043	

B

w/o	+ NaHCO$_3$	w/o	+ NaHCO$_3$	w/o	+ NaHCO$_3$
COL		PB 027-133		PB 017-037	
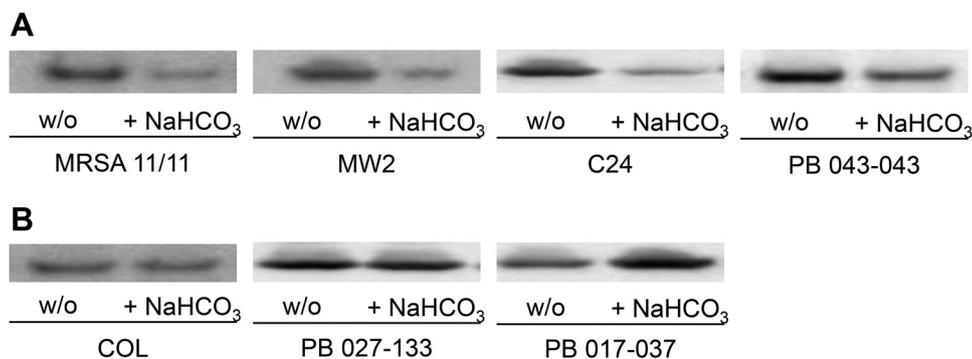
FIG 4 Membrane PrsA protein content in MRSA strains grown in the presence and absence of NaHCO$_3$. (A) NaHCO$_3$-responsive strains. (B) Nonresponsive strains. Strains were grown in CA-MHB plus 100 mM Tris (w/o) or CA-MHB plus 100 mM Tris plus 44 mM NaHCO$_3$ (+ NaHCO$_3$) in the presence of 0.5× MIC oxacillin. Proteins are the membrane fraction extracted from log-phase cells.

sion of *asp23*, a surrogate reporter for *sigB* promoter activity (31). Interestingly, in both NaHCO$_3$-responsive and -nonresponsive strains, *asp23* expression was substantially and uniformly repressed in the presence of NaHCO$_3$ (data not shown). To confirm the phenotypic outcome of such globally repressed SigB activity, carotenoid content was measured in NaHCO$_3$-responsive and -nonresponsive strains and was found to be universally reduced by NaHCO$_3$ exposures (Fig. 5A).

Since carotenoids interact with flotillin within the CM to form FMMs (19), we determined whether NaHCO$_3$ also impacts the flotillin component of FMMs by assessing the expression of *floA*. Interestingly, *floA* gene expression was increased in all strains in the presence of NaHCO$_3$ (Fig. 5B). Combined with the reduced carotenoid content data described above, the latter data indicate that NaHCO$_3$ is inducing a proportionality disequilibrium in the formation of stable FMMs (i.e, reduced carotenoids plus increased *floA* expression).

DISCUSSION

Previously, we identified a novel MRSA phenomenon, termed NaHCO$_3$ responsiveness, wherein a relatively large proportion of MRSA strains displayed increased susceptibility to standard β-lactam antibiotics in the presence of NaHCO$_3$ (11, 12). Thus, ∼75% and ∼33% of a collection of 58 recent MRSA bloodstream isolates were significantly more susceptible *in vitro* to cefazolin and oxacillin, respectively, in the presence versus absence of NaHCO$_3$ (12). We also linked this responsiveness phenomenon to the reduced expression of two genes involved in the maintenance of the MRSA phenotype, *mecA* and *sarA* (11). These genetic impacts translated into reduced PBP2a production in such NaHCO$_3$-responsive MRSA in the presence of NaHCO$_3$.

In the present study, we further investigated additional mechanisms potentially involved in NaHCO$_3$–β-lactam responsiveness, in eight representative strains, focusing on the impact of NaHCO$_3$ upon both *mecA-blaZ* coregulation and key genes involved in the maturation functionality of PBP2a; each of these events is critical in determining ultimate MRSA β-lactam resistance.

Several interesting observations emerged from these investigations. First, in the presence of oxacillin induction, NaHCO$_3$ substantially reduced the expression of both *mecA* and *blaZ* only in NaHCO$_3$-responsive strains. Thus, it seems clear that in NaHCO$_3$-responsive strains, NaHCO$_3$ impacts both limbs of the *mecA-blaZ* coregulatory axis important in the maintenance of the MRSA phenotype (20). Also, our prior studies, utilizing a latex agglutination assay, showed that NaHCO$_3$ could reduce overall production of PBP2a in NaHCO$_3$-responsive versus -nonresponsive MRSA (11). Similar PBP2a production differences were confirmed using this same assay in additional strains as well as with a more precise Western blot assay for CM localization of this PBP.

A

Carotenoid Production

B
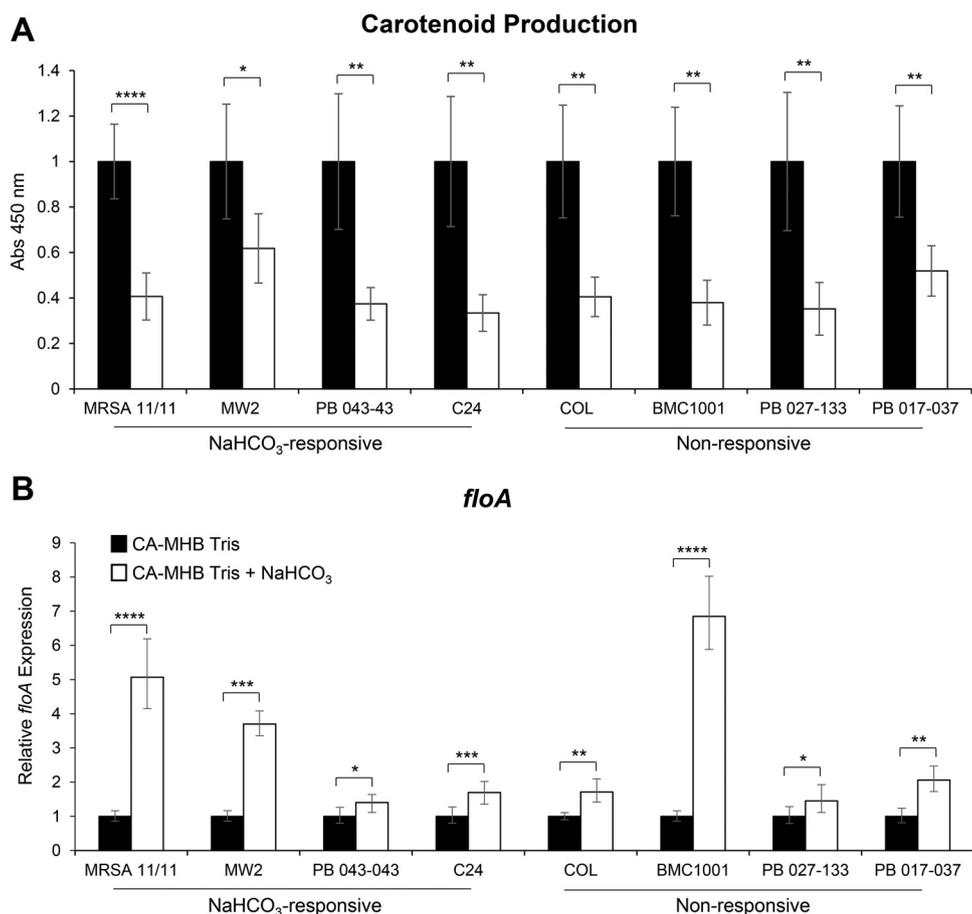
floA

FIG 5 Impact of NaHCO$_3$ on FMM-associated factors. (A and B) Carotenoid production (A) and *floA* gene expression (B) in MRSA strains grown in the presence and absence of NaHCO$_3$. *floA* gene expression was assessed by qRT-PCR using RNA extracted from log-phase cells (OD$_{600}$, 0.4) in the presence of 0.5× MIC oxacillin. Black bars indicate growth in CA-MHB with 100 mM Tris, white bars indicate growth in CA-MHB with 100 mM Tris plus 44 mM NaHCO$_3$ for all graphs, and error bars indicate the C_T standard deviations. Student's t test was used for statistical analyses. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

Second, we established that NaHCO$_3$ exposure *in vitro* had a rather profound dampening effect on the expression of several key genes involved in PBP2a maturation. For example, the two-component regulatory system, VraSR, and the VraSR-regulated chaperone, PrsA, are known to work in concert; dysfunction in this circuit appears to underlie the see-saw effect, wherein daptomycin-resistant MRSA strains become hypersusceptible to β-lactams (21, 22). Interestingly, such see-saw strains actually demonstrate increased expression of both *vraSR* and *prsA* as well as the accumulation of mutations in *mprF* (21, 23). The collateral effects of the latter genetic changes are the decreased production of PrsA in the CM and concomitant increased extracellular secretion of PrsA, with a resultant decreased localization of PBP2a in the CM (21). In contrast, in NaHCO$_3$-responsive MRSA, there appears to be a totally different mechanism involved in β-lactam hypersusceptibility. Thus, we did not observe excess secretion of PrsA in NaHCO$_3$-responsive strains that could explain the decreased PrsA levels within the CM. These results suggest that NaHCO$_3$ is not directly affecting PrsA functionality but rather more directly affecting overall PrsA protein levels. NaHCO$_3$ reduces the expression of both *vraSR* and *prsA*, thereby decreasing the overall production and CM localization of the PrsA chaperone, potentially impairing the ultimate maturation and functionality of PBP2a within the CM.

Third, NaHCO$_3$ reduced *pbp4* expression selectively in NaHCO$_3$-responsive strains. Importantly, *pbp4* is a nonessential PBP that functions as an auxiliary transpeptidase

during cell wall synthesis (32). Of relevance to the present study, deletion of *pbp4* in certain community-acquired MRSA strains (e.g., USA300 and MW2, both NaHCO$_3$ responsive) resulted in a 16-fold increased susceptibility to oxacillin, while this deletion had no effect on oxacillin MICs in strain COL (NaHCO$_3$ nonresponsive) (26). Further, in USA300 and MW2, *pbp4* mutants exposed to oxacillin exhibited substantially less highly cross-linked cell wall peptidoglycan, indicating a cooperative action between PBP2a and PBP4 in cell wall synthesis during β-lactam exposure. As demonstrated by our data, NaHCO$_3$ reduces both the transcription of *pbp4* and overall production of PBP2a, which likely leads to a net decrease in highly cross-linked peptidoglycan in NaHCO$_3$-responsive strains. Although little is known about the regulation of *pbp4* expression (33), deletion of *pbp4* results in a decrease in *pbp2* transcription (26). Recent analyses of the transcriptomes of NaHCO$_3$-responsive strains by RNA sequencing from our laboratory confirm that NaHCO$_3$ downregulates *pbp2* expression (unpublished data); this suggests that NaHCO$_3$ is acting on an upstream regulator of both *pbp2* and *pbp4*. Interestingly, PBP2 is one of only three known enzymes with transglycosylase activity in *S. aureus* (34), indicating that NaHCO$_3$ has additional impacts on peptidoglycan transglycosylation in responsive MRSA strains. Further work is needed to understand the specific impact of NaHCO$_3$ on the regulation of the overall cadre of genes involved in peptidoglycan biosynthesis as well as the impact of NaHCO$_3$ on the abundance of highly cross-linked peptidoglycan muropeptides within the MRSA cell wall. These investigations are ongoing in our laboratories.

Lastly, in addition to strain-specific and selective impacts described above in NaHCO$_3$-responsive versus -nonresponsive MRSA, NaHCO$_3$ also had several, more global effects in all eight prototype strains that could impact CM physiology. For example, NaHCO$_3$ reduced the activity of the global stress response regulator, SigB (35, 36), in all strains studied. In *S. aureus*, SigB upregulates the expression of the operon *crtOPQMN*, which is responsible for the biosynthesis of the triterpenoid carotenoid pigment staphyloxanthin (17, 37). Staphyloxanthin, in turn, is a crucial factor in the formation of FMMs, which create the scaffolding for oligomerization of PBP2a within the CM (19). Interestingly, disruption of staphyloxanthin synthesis by clinical statin agents in *S. aureus* leads to increased susceptibility to β-lactams (19). As expected, based on the NaHCO$_3$ repression of SigB activity, carotenoid production was concomitantly reduced in all strains tested. Conversely, NaHCO$_3$ exposure resulted in increased *floA* expression in all strains tested, highlighting the possibility that NaHCO$_3$ is disrupting the normal equilibrium of two key factors necessary for stable FMM formation (i.e., relative proportional contents of carotenoids and FloA). This disequilibrium could contribute to the NaHCO$_3$-responsive phenotype when other observed alterations in PBP2a maturation are also present.

There are several limitations to our investigations. For example, we only studied a relatively small number of MRSA strains ($n = 8$). Additionally, we carefully selected only a specific cadre of genes to study that have been well characterized as to their known impacts on PBP2a regulation, production, and functionality. Further, we have not yet investigated how changes in gene expression impact cell wall morphology and FMM composition/organization. Moreover, the more global genetic impacts of NaHCO$_3$ in MRSA await more extensive analyses, such as whole-genome sequencing and RNA sequencing, studies that are in progress. Finally, genetic swapping studies between NaHCO$_3$-responsive and -nonresponsive strains will be required to more precisely define the role of specific PBP2a genotype species in the β-lactam sensitization phenomenon related to NaHCO$_3$ exposures (38). Importantly, understanding the mechanism(s) of NaHCO$_3$ responsiveness may help guide future clinical microbiologic laboratory screening strategies as well as the repurposing of treatment practices for MRSA infections, resulting in improved patient outcomes using β-lactam-based therapies.

Figure 6 represents one proposed pathway for the overall impact of NaHCO$_3$ on key MRSA genotypes and phenotypes, based on our current investigations.

In summary, NaHCO$_3$ was found to have profound effects on PBP2a that were
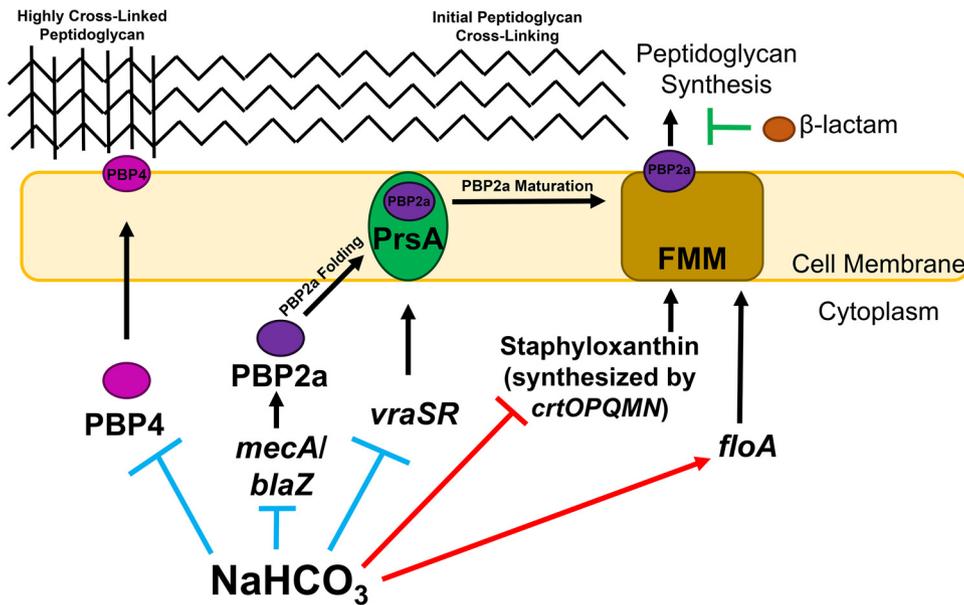
FIG 6 Schematic overview of the impact of NaHCO₃ on PBP2a maturation/function and peptidoglycan biosynthesis. Impacts specific to NaHCO₃-responsive strains (blue inhibition symbols) include the repression of PBP2a protein production, *pbp4* gene expression, *vraSR* gene expression, and PrsA protein production. Impacts of NaHCO₃ on all strains tested include repression of carotenoid production (red inhibition symbol) and increased *floA* gene expression (red enhancement symbol), potentially resulting in less stable functional membrane microdomains (FMMs). Anticipated collateral impact on NaHCO₃-responsive strains is an overall decrease in PBP2a mediated peptidoglycan synthesis and the generation of highly cross-linked peptidoglycan, resulting in increased susceptibility to β-lactam antibiotics.

specific to NaHCO₃-responsive strains (e.g., *mecA* and *blaZ* expression, PBP2a protein production and CM localization, and expression of genes involved in PBP2a maturation and functionality). In contrast, NaHCO₃ impacted certain genotypes-phenotypes more globally in both NaHCO₃-responsive and -nonresponsive MRSA (i.e., SigB activity, downstream carotenoid production, and *floA* expression). The latter data indicate that certain NaHCO₃-mediated impacts are themselves involved and necessary in the collective NaHCO₃-responsive outcome in MRSA, but they are not individually sufficient to cause this overall phenotype without additional perturbations in PBP2a production, maturation, and functionality (Fig. 6).

MATERIALS AND METHODS

Bacterial strains and media. All MRSA strains used in this study were initially isolated from patients with invasive clinical infections: MRSA 11/11, MW2, PB 043-043, C24, COL, BMC1001, PB 027-133, and PB 017-037 (12, 22, 39–41). Four of these strains (MRSA 11/11, MW2, BMC1001, and COL) were selected based on their inclusion in a prior study on the NaHCO₃-responsive phenomenon (11). The other four strains (PB 043-043, C24, PB 027-133, and PB 017-037) were selected from a larger screen of NaHCO₃-responsive MRSA (12) based on their oxacillin and cefazolin MICs in the presence/absence of NaHCO₃ and their clonal complex (CC) types. The CC types of 7/8 isolates represent genotypes in current widespread clinical circulation (i.e., CC types 5 and 8) (see Table S1 in the supplemental material) (42). MRSA 11/11, MW2, PB 043-043, C24, BMC1001, PB 027-133, and PB 017-037 are all β-lactamase positive; COL is naturally β-lactamase negative. MICs were determined, in the presence or absence of NaHCO₃, by standard CLSI methods (Table S1). MICs for MRSA 11/11, MW2, COL, and BMC1001 have been previously reported (11).

For all experiments, strains were grown in ambient air at 37°C in cation-adjusted Mueller-Hinton Broth (CA-MHB; Difco) plus 100 mM Tris (to stabilize the pH at ~7.3 ± 0.1) with or without 44 mM NaHCO₃ (the optimal concentration to disclose the NaHCO₃-responsive phenotype *in vitro* and *ex vivo*, reflective of deep-tissue HCO₃ levels [11–13, 43]). For oxacillin exposure experiments, 0.5× MIC oxacillin was used for a given strain in the specified growth medium, with 2% NaCl incorporated into testing media (Table S1).

MIC assays. The MICs of oxacillin were determined by broth microdilution according to CLSI guidelines in specified media, as previously described (9, 11, 44). All MICs are the mode of at least six independent determinations.

PBP2a latex agglutination assays. A semiquantitative, rapid, and reliable latex agglutination method (Seiken, Tokyo, Japan) was used to approximate total PBP2a production, as previously described (11). Agglutination results were scored as high ($+++$), moderate ($++$), low ($+$), or negative ($-$) based on the presence or absence of an overt agglutination pattern, corresponding to the total amount of PBP2a in the sample. *S. aureus* ATCC 43300 (MRSA; PBP2a positive) and ATCC 25923 (MSSA; PBP2a negative) were used as positive and negative controls, respectively, in all assays. The grading of agglutination assays was performed blindly as to strain identifications by one of the investigators (S. C. Ersoy).

Membrane (CM) protein extraction. Cells were grown to stationary phase (PBP2a) or log phase (optical density at 600 nm [OD_{600}], 0.5; for PrsA) in CA-MHB plus 100 mM Tris plus $0.5\times$ MIC oxacillin, with or without 44 mM $NaHCO_3$ (Table S1), and their centrifuged pellets were then resuspended in phosphate-buffered saline (PBS), pH 7.3. Thereafter, 1 ml of resuspended pellets was incubated with 10 μl each of DNase (Ambion, Invitrogen), RNase (Thermo Fisher Scientific), and Halt protease inhibitor cocktail (Thermo Fisher Scientific) for 30 min at 37°C. Cells were then mixed with glass beads and disrupted using a FastPrep cell disrupter. Disrupted cells were centrifuged for 20 min at 4°C and 13,000 rpm, and the supernatant was ultracentrifuged for 2 h at 4°C and 15,000 rpm to collect total CM proteins. The CM protein pellets were resuspended in PBS, and protein concentrations were quantified by Bradford protein assay. Isolated protein extracts were stored at -80°C.

Western blotting for PBP2a and PrsA protein expression. Forty micrograms of CM protein extract was separated on a 4 to 12% Bis-Tris gel (Invitrogen) and then blotted onto a nitrocellulose membrane (Amersham). Total protein loading was confirmed by staining with 0.25% Ponceau reagent (Fig. S1). 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 1:2,500 and detected with an anti-chicken IgY cross-absorbed secondary antibody, horseradish peroxidase (Thermo Fisher Scientific), diluted 1:5,000. In parallel studies, PrsA was probed with a chicken anti-PrsA antibody diluted 1:2,500 (21). Labeled proteins were imaged using a c400 imager (Azure Biosystems). Western blotting for PrsA in strain BMC1001 could not be performed due to a single-nucleotide deletion within its *prsA* coding region, causing a frameshift mutation resulting in a premature stop codon (*prsA* g. 796_798del; p. L272X).

Isolation of RNA and qRT-PCR analysis. RNA was isolated from strains as detailed previously (11). Quantitative real-time PCR (qRT-PCR) was performed using primers for *blaZ*, *prsA*, *vrsASR*, *pbp4*, *floA*, *mecA*, and *gyrB* as previously described (15, 25, 45–47); these primers are listed in Table S2. The *gyrB* gene was used as a housekeeping gene for transcript normalization (verification of the adequacy of *gyrB* as a reproducible control for these assays was independently done using *rpoB* in parallel experiments [data not shown]) (48). Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method from two independent biological replicates performed in triplicate on at least 2 separate runs for each strain/condition. Data are presented as the relative gene expression in CA-MHB plus 100 mM Tris plus 44 mM $NaHCO_3$ normalized to the relative gene expression in CA-MHB plus 100 mM Tris for each strain, where expression in CA-MHB 100 mM Tris was set to 1.0. A Student's *t* test was used for all statistical analyses.

Carotenoid production assay. To quantify relative levels of staphyloxanthin production, carotenoids were isolated by methanol extraction as previously described (37). Briefly, cells were grown overnight in specified medium, pelleted, and washed twice with PBS. Methanol was added to pellets at a volume that was normalized to total cell weight for each sample (0.0625 g cell weight/ml of methanol). Samples were vortexed vigorously for 10 min to allow carotenoid extraction from the cell pellet into methanol. Methanol absorbance was measured at the OD_{450}. A higher absorbance reading indicates greater carotenoid production. The absorbance for cells grown in CA-MHB plus 100 mM Tris plus 44 mM $NaHCO_3$ was normalized to the absorbance in CA-MHB plus 100 mM Tris for each strain, where CA-MHB plus 100 mM Tris equals 1.0. A Student's *t* test was used for all statistical analyses.

Data availability. The whole-genome shotgun project for strains BMC1001, COL, MRSA 11/11, and MW2 have been deposited at DDBJ/ENA/GenBank as a BioProject (PRJNA697971) under the accession numbers JAFFHU000000000, JAFFHV000000000, JAFFHW000000000, and JAFFHX000000000. The versions described in this paper are versions JAFFHU010000000, JAFFHV010000000, JAFFHW010000000, and JAFFHX010000000.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.8 MB.

REFERENCES

1. Murray R. 2005. *Staphylococcus aureus* infective endocarditis: diagnosis and management guidelines. Intern Med J 35:S25–S44. https://doi.org/10.1111/j.1444-0903.2005.00978.x.

2. Grundmann H, Aires-de-Sousa M, Boyce J, Tiemersma E. 2006. Emergence and resurgence of meticillin-resistant *Staphylococcus aureus* as a public-health threat. Lancet 368:874–885. https://doi.org/10.1016/S0140-6736(06)68853-3.

3. Tong SY, Davis JS, Eichenberger E, Holland TL, Fowler VG. 2015. *Staphylococcus aureus* infections: epidemiology, pathophysiology, clinical manifestations, and management. Clin Microbiol Rev 28:603–661. https://doi.org/10.1128/CMR.00134-14.

4. Dien Bard J, Hindler JA, Gold HS, Limbago B. 2014. Rationale for eliminating *Staphylococcus* breakpoints for β-lactam agents other than penicillin, oxacillin or cefoxitin, and ceftaroline. Clin Infect Dis 58:1287–1296. https://doi.org/10.1093/cid/ciu043.

5. Purrello S, Garau J, Giamarellos E, Mazzei T, Pea F, Soriano A, Stefani S. 2016. Methicillin-resistant *Staphylococcus aureus* infections: a review of the currently available treatment options. J Glob Antimicrob Resist 7:178–186. https://doi.org/10.1016/j.jgar.2016.07.010.

6. Edwards B, Andini R, Esposito S, Grossi P, Lew D, Mazzei T, Novelli A, Soriano A, Gould I. 2014. Treatment options for methicillin-resistant *Staphylococcus aureus* (MRSA) infection: where are we now? J Glob Antimicrob Resist 2:133–140. https://doi.org/10.1016/j.jgar.2014.03.009.

7. Goetghebeur M, Landry P-A, Han D, Vicente C. 2007. Methicillin-resistant *Staphylococcus aureus*: a public health issue with economic consequences. Can J Infect Dis Med Microbiol 18:27–34. https://doi.org/10.1155/2007/253947.

8. Datta P, Gulati N, Singla N, Vasdeva HR, Bala K, Chander J, Gupta V. 2011. Evaluation of various methods for the detection of meticillin-resistant *Staphylococcus aureus* strains and susceptibility patterns. J Med Microbiol 60:1613–1616. https://doi.org/10.1099/jmm.0.032219-0.

9. Weinstein MP, Patel JB, Campeau S, Eliopoulos GM, Galas MF, Humphries RM, Jenkins SG, Li JSL, Limbago B, Mathers AJ, Mazzulli T, Patel R, Richter SS, Satlin M, Swenson JM, Zimmer BL. 2018. Performance standards for antimicrobial susceptibility testing. Clinical and Laboratory Standards Institute, Wayne, PA.

10. Chen L, Mediavilla JR, Oliveira DC, Willey BM, de Lencastre H, Kreiswirth BN. 2009. Multiplex real-time PCR for rapid staphylococcal cassette chromosome *mec* typing. J Clin Microbiol 47:3692–3706. https://doi.org/10.1128/JCM.00766-09.

11. Ersoy SC, Abdelhady W, Li L, Chambers HF, Xiong YQ, Bayer AS. 2019. Bicarbonate resensitization of methicillin-resistant *Staphylococcus aureus* to β-Lactam antibiotics. Antimicrob Agents Chemother 63:e00496-19. https://doi.org/10.1128/AAC.00496-19.

12. Ersoy SC, Otmishi M, Milan VT, Li L, Pak Y, Mediavilla J, Chen L, Kreiswirth B, Chambers HF, Proctor RA, Xiong YQ, Fowler VG, Bayer AS. 2020. Scope and predictive genetic/phenotypic signatures of 'bicarbonate [NaHCO3]-responsiveness' and β-Lactam sensitization among methicillin-resistant *Staphylococcus aureus* (MRSA). Antimicrob Agents Chemother 64:e02445-19. https://doi.org/10.1128/AAC.02445-19.

13. Rose WE, Bienvenida AM, Xiong YQ, Chambers HF, Bayer AS, Ersoy SC. 2019. Ability of bicarbonate supplementation to sensitize selected methicillin-resistant *Staphylococcus aureus* (MRSA) strains to β-Lactam antibiotics in an ex vivo simulated endocardial vegetation model. Antimicrob Agents Chemother 64:e02072-19. https://doi.org/10.1128/AAC.02072-19.

14. Farha MA, French S, Stokes JM, Brown ED. 2018. Bicarbonate alters bacterial susceptibility to antibiotics by targeting the proton motive force. ACS Infect Dis 4:382–390. https://doi.org/10.1021/acsinfecdis.7b00194.

15. Li L, Cheung A, Bayer AS, Chen L, Abdelhady W, Kreiswirth BN, Yeaman MR, Xiong YQ. 2016. The global regulon *sarA* regulates beta-lactam antibiotic resistance in methicillin-resistant *Staphylococcus aureus in vitro* and in endovascular infections. J Infect Dis 214:1421–1429. https://doi.org/10.1093/infdis/jiw386.

16. Bischoff M, Entenza J, Giachino P. 2001. Influence of a functional *sigB* operon on the global regulators *sar* and *agr* in *Staphylococcus aureus*. J Bacteriol 183:5171–5179. https://doi.org/10.1128/jb.183.17.5171-5179.2001.

17. Katzif S, Lee E-H, Law AB, Tzeng Y-L, Shafer WM. 2005. CspA regulates pigment production in *Staphylococcus aureus* through a SigB-dependent mechanism. J Bacteriol 187:8181–8184. https://doi.org/10.1128/JB.187.23.8181-8184.2005.

18. Foster TJ. 2019. Can β-lactam antibiotics be resurrected to combat MRSA? Trends Microbiol 27:26–38. https://doi.org/10.1016/j.tim.2018.06.005.

19. García-Fernández E, Koch G, Wagner RM, Fekete A, Stengel ST, Schneider J, Mielich-Süss B, Geibel S, Markert SM, Stigloher C, Lopez D. 2017. Membrane microdomain disassembly inhibits MRSA antibiotic resistance. Cell 171:1354–1367. https://doi.org/10.1016/j.cell.2017.10.012.

20. Hackbarth CJ, Chambers HF. 1993. *blaI* and *blaR1* regulate beta-lactamase and PBP 2a production in methicillin-resistant *Staphylococcus aureus*. Antimicrob Agents Chemother 37:1144–1149. https://doi.org/10.1128/aac.37.5.1144.

21. Renzoni A, Kelley WL, Rosato RR, Martinez MP, Roch M, Fatouraei M, Haeusser DP, Margolin W, Fenn S, Turner RD, Foster SJ, Rosato AE. 2017. Molecular bases determining daptomycin resistance-mediated resensitization to β-lactams (seesaw effect) in methicillin-resistant *Staphylococcus aureus*. Antimicrob Agents Chemother 61:e01634-16. https://doi.org/10.1128/AAC.01634-16.

22. Yang S-J, Xiong YQ, Boyle-Vavra S, Daum R, Jones T, Bayer AS. 2010. Daptomycin-oxacillin combinations in treatment of experimental endocarditis caused by daptomycin-nonsusceptible strains of methicillin-resistant *Staphylococcus aureus* with evolving oxacillin susceptibility (the "seesaw effect"). Antimicrob Agents Chemother 54:3161–3169. https://doi.org/10.1128/AAC.00487-10.

23. Mehta S, Cuirolo AX, Plata KB, Riosa S, Silverman JA, Rubio A, Rosato RR, Rosato AE. 2012. VraSR two-component regulatory system contributes to *mprF*-mediated decreased susceptibility to daptomycin in *in vivo*-selected clinical strains of methicillin-resistant *Staphylococcus aureus*. Antimicrob Agents Chemother 56:92–102. https://doi.org/10.1128/AAC.00432-10.

24. Jousselin A, Renzoni A, Andrey DO, Monod A, Lew DP, Kelley WL. 2012. The post-translocational chaperone lipoprotein PrsA is involved in both glycopeptide and oxacillin resistance in *Staphylococcus aureus*. Antimicrob Agents Chemother 56:3629–3640. https://doi.org/10.1128/AAC.06264-11.

25. Jousselin A, Manzano C, Biette A, Reed P, Pinho M, Rosato A, Kelley WL, Renzoni A. 2015. The *Staphylococcus aureus* chaperone PrsA is a new auxiliary factor of oxacillin resistance affecting penicillin-binding protein 2A. Antimicrob Agents Chemother 60:1656–1666. https://doi.org/10.1128/AAC.02333-15.

26. Memmi G, Filipe SR, Pinho MG, Fu Z, Cheung A. 2008. *Staphylococcus aureus* PBP4 is essential for β-lactam resistance in community-acquired methicillin-resistant strains. Antimicrob Agents Chemother 52:3955–3966. https://doi.org/10.1128/AAC.00049-08.

27. Łeski TA, Tomasz A. 2005. Role of penicillin-binding protein 2 (PBP2) in the antibiotic susceptibility and cell wall cross-linking of *Staphylococcus aureus*: evidence for the cooperative functioning of PBP2, PBP4, and PBP2A. J Bacteriol 187:1815–1824. https://doi.org/10.1128/JB.187.5.1815-1824.2005.

28. Ersoy SC, Chambers HF, Proctor RA, Rosato AE, Mishra NN, Xiong YQ, Bayer AS. 2021. Impact of bicarbonate on PBP2a production, maturation, and functionality in methicillin-resistant *Staphylococcus aureus*. 4th Annu Texas Med Center Antimicrob Resist Steward Conf, poster 30.

29. Reed P, Atilano ML, Alves R, Hoiczyk E, Sher X, Reichmann NT, Pereira PM, Roemer T, Filipe SR, Pereira-Leal JB, Ligoxygakis P, Pinho MG. 2015. *Staphylococcus aureus* survives with a minimal peptidoglycan synthesis machine but sacrifices virulence and antibiotic resistance. PLoS Pathog 11:e1004891. https://doi.org/10.1371/journal.ppat.1004891.

30. Cheung AL, Zhang G. 2002. Global regulation of virulence determinants in *Staphylococcus aureus* by the SarA protein family. Front Biosci 7:d1825–d1842. https://doi.org/10.2741/cheung.

31. Giachino P, Engelmann S, Bischoff M. 2001. ςB activity depends on RsbU in *Staphylococcus aureus*. J Bacteriol 183:1843–1852. https://doi.org/10.1128/JB.183.6.1843-1852.2001.

32. da Costa TM, de Oliveira CR, Chambers HF, Chatterjee SS. 2018. PBP4: a new perspective on *Staphylococcus aureus* β-lactam resistance. Microorganisms 6:57. https://doi.org/10.3390/microorganisms6030057.

33. Chatterjee SS, Chen L, Gilbert A, Da Costa TM, Nair V, Datta SK, Kreiswirth BN, Chambers HF. 2017. PBP4 mediates β-lactam resistance by altered function. Antimicrob Agents Chemother 61:e00932-17. https://doi.org/10.1128/AAC.00932-17.

34. Reed P, Veiga H, Jorge AM, Terrak M, Pinho MG. 2011. Monofunctional transglycosylases are not essential for Staphylococcus aureus cell wall synthesis. J Bacteriol 193:2549–2556. https://doi.org/10.1128/JB.01474-10.

35. Chan PF, Foster SJ, Ingham E, Clements MO. 1998. The Staphylococcus aureus alternative sigma factor ςB controls the environmental stress response but not starvation survival or pathogenicity in a mouse abscess model. J Bacteriol 180:6082–6089. https://doi.org/10.1128/.180.23.6082-6089.1998.

36. Gertz S, Engelmann S, Schmid R, Ziebandt A-K, Tischer K, Scharf C, Hacker J, Hecker M. 2000. Characterization of the ςB regulon in Staphylococcus aureus. J Bacteriol 182:6983–6991. https://doi.org/10.1128/jb.182.24.6983-6991.2000.

37. Mishra NN, Liu GY, Yeaman MR, Nast CC, Proctor RA, McKinnell J, Bayer AS. 2011. Carotenoid-related alteration of cell membrane fluidity impacts Staphylococcus aureus susceptibility to host defense peptides. Antimicrob Agents Chemother 55:526–531. https://doi.org/10.1128/AAC.00680-10.

38. Harrison EM, Ba X, Coll F, Blane B, Restif O, Carvell H, Köser CU, Jamrozy D, Reuter S, Lovering A, Gleadall N, Bellis KL, Uhlemann A-C, Lowy FD, Massey RC, Grilo IR, Sobral R, Larsen J, Rhod Larsen A, Vingsbo Lundberg C, Parkhill J, Paterson GK, Holden MTG, Peacock SJ, Holmes MA. 2019. Genomic identification of cryptic susceptibility to penicillins and β-lactamase inhibitors in methicillin-resistant Staphylococcus aureus. Nat Microbiol 4:1680–1691. https://doi.org/10.1038/s41564-019-0471-0.

39. Voyich JM, Braughton KR, Sturdevant DE, Whitney AR, Saïd-Salim B, Porcella SF, Long RD, Dorward DW, Gardner DJ, Kreiswirth BN, Musser JM, DeLeo FR. 2005. Insights into mechanisms used by Staphylococcus aureus to avoid destruction by human neutrophils. J Immunol 175:3907–3919. https://doi.org/10.4049/jimmunol.175.6.3907.

40. Murthy MH, Olson ME, Wickert RW, Fey PD, Jalali Z. 2008. Daptomycin non-susceptible methicillin-resistant Staphylococcus aureus USA 300 isolate. J Med Microbiol 57:1036–1038. https://doi.org/10.1099/jmm.0.2008/000588-0.

41. Bratu S, Eramo A, Kopec R, Coughlin E, Ghitan M, Yost R, Chapnick EK, Landman D, Quale J. 2005. Community-associated methicillin-resistant Staphylococcus aureus in hospital nursery and maternity units. Emerg Infect Dis 11:808–813. https://doi.org/10.3201/eid1106.040885.

42. Dupper AC, Sullivan MJ, Chacko KI, Mishkin A, Ciferri B, Kumaresh A, Berbel Caban A, Oussenko I, Beckford C, Zeitouni NE. 2020. Blurred molecular epidemiological lines between the two dominant methicillin-resistant Staphylococcus aureus clones p ofz302. Oxford University Press, Oxford, United Kingdom.

43. Fenn WO. 1928. The carbon dioxide dissociation curve of nerve and muscle. Am J Physiol Legacy Content 85:207–223. https://doi.org/10.1152/ajplegacy.1928.85.2.207.

44. Cockerill FR. 2012. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically: approved standard. Clinical and Laboratory Standards Institute, Wayne, PA.

45. Dumitrescu O, Choudhury P, Boisset S, Badiou C, Bes M, Benito Y, Wolz C, Vandenesch F, Etienne J, Cheung AL, Bowden MG, Lina G. 2011. β-Lactams interfering with PBP1 induce panton-valentine leukocidin expression by triggering sarA and rot global regulators of Staphylococcus aureus. Antimicrob Agents Chemother 55:3261–3271. https://doi.org/10.1128/AAC.01401-10.

46. Seidl K, Chen L, Bayer AS, Hady WA, Kreiswirth BN, Xiong YQ. 2011. Relationship of agr expression and function with virulence and vancomycin treatment outcomes in experimental endocarditis due to methicillin-resistant Staphylococcus aureus. Antimicrob Agents Chemother 55:5631–5639. https://doi.org/10.1128/AAC.05251-11.

47. Mielich-Süss B, Wagner RM, Mietrach N, Hertlein T, Marincola G, Ohlsen K, Geibel S, Lopez D. 2017. Flotillin scaffold activity contributes to type VII secretion system assembly in Staphylococcus aureus. PLoS Pathog 13: e1006728. https://doi.org/10.1371/journal.ppat.1006728.

48. Sihto H-M, Tasara T, Stephan R, Johler S. 2014. Validation of reference genes for normalization of qPCR mRNA expression levels in Staphylococcus aureus exposed to osmotic and lactic acid stress conditions encountered during food production and preservation. FEMS Microbiol Lett 356:134–140. https://doi.org/10.1111/1574-6968.12491.