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Staphylococcus aureus PBP4 Is Essential for β-Lactam Resistance in Community-Acquired Methicillin-Resistant Strains▽

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Recent cases of infections caused by community-acquired methicillin-resistant *Staphylococcus aureus* (MRSA) (CA-MRSA) strains in healthy individuals have raised concerns worldwide. CA-MRSA strains differ from hospital-acquired MRSAs by virtue of their genomic background and increased virulence in animal models. Here, we show that in two common CA-MRSA isolates, USA300 and MW2 (USA400), a loss of penicillin binding protein 4 (PBP4) is sufficient to cause a 16-fold reduction in oxacillin and nafcillin resistance, thus demonstrating that *mecA*, encoding PBP2A, is not the sole determinant of methicillin resistance in CA-MRSA. The loss of PBP4 was also found to severely affect the transcription of PBP2 in cells after challenge with oxacillin, thus leading to a significant decrease in peptidoglycan cross-linking. Autolysis, which is commonly associated with the killing mechanism of penicillin and β-lactams, does not play a role in the reduced resistance phenotype associated with the loss of PBP4. We also showed that cefoxitin, a semisynthetic β-lactam that binds irreversibly to PBP4, is synergistic with oxacillin in killing CA-MRSA strains, including clinical CA-MRSA isolates. Thus, PBP4 represents a major target for drug rediscovery against CA-MRSA, and a combination of cefoxitin and synthetic penicillins may be an effective therapy for CA-MRSA infections.

Methicillin-resistant *Staphylococcus aureus* (MRSA), the most common cause of nosocomial infections, was previously described to be an opportunistic pathogen (1). However, the incidence of community-acquired MRSA (CA-MRSA) infections has substantially increased over the last 5 years in healthy individuals without any known risk factors (4–7, 13, 20, 21, 31, 32, 42, 56). Not only is this medically relevant, but the fact that methicillin resistance has originated in strains not associated with nosocomial environments and/or antibiotic exposure is astonishing and warrants further study. USA300 and MW2 (USA400), representing two distinct clinical isolates, are the predominant CA-MRSA strains found in the United States (3, 21). Although initially seen in cutaneous infections, USA300 has now become a major cause of sepsis and prosthetic joint infections, with limited therapeutic options (14, 33, 52, 54). Although hospital-acquired MRSA (HA-MRSA) and CA-MRSA strains have similar genomic backgrounds, they do carry substantial differences, which may explain why community-acquired strains are significantly more virulent than their hospital-acquired counterparts in a mouse model of *S. aureus* infection, as they are able to cause a greater level of pathology in major vital organs, more resistant to killing by human polymorphonuclear leukocytes, and capable of causing greater host cell lysis (57).

Resistance to a greater number of antibiotics has occurred in *S. aureus* isolates worldwide. Besides common resistance to methicillin and β-lactams in general, *S. aureus* has also become resistant to drugs of last resort such as vancomycin, linezolid, and daptomycin (14, 25, 27, 49, 51, 54). All *S. aureus* isolates, both methicillin-sensitive and -resistant strains, carry three high-molecular-weight penicillin binding proteins (PBPs), PBP1, PBP2, and PBP3, to which most β-lactam antibiotics bind. The β-lactam antibiotics generally target the transpeptidase domain of PBPs, which leads to a reduction in cell wall cross-linking and, hence, a loss of cell wall integrity (39). PBP4, the single low-molecular-weight PBP, which has been shown to have a low affinity for most β-lactams, is unique among low-molecular-weight PBPs in that it possesses transpeptidase and carboxypeptidase activities (16–18, 34).

Methicillin resistance is achieved by the acquisition of another high-molecular-weight PBP, namely, PBP2A, encoded by *mecA*; this is situated in the chromosome in a genomic island designated staphylococcal cassette chromosome *mec* (SCC*mec*). Unlike innate PBPs, PBP2A has a remarkably low affinity for all β-lactams.

Early data have linked PBP4 to low-level methicillin resistance in strains lacking *mecA* (11, 18, 22, 23). Interestingly, a loss of PBP4 in MRSA prototypic strain COL had relatively little effect on β-lactam resistance (30). However, the role of PBP4 in methicillin resistance and β-lactam resistance in general in CA-MRSA strains has not yet been evaluated.

Here, we showed that a loss of *pbp4* in CA-MRSA strains MW2 and USA300 leads to a dramatic decrease in oxacillin and nafcillin resistance, while the same deletion does not confer a similar phenotype in the HA-MRSA strains COL and N315 and vancomycin-intermediate *S. aureus* (VISA) strain Mu50. We also discovered that a loss of PBP4 has a dramatic impact on PBP2 transcription in cells challenged with oxacillin in CA-MRSA strain MW2 but not in HA-MRSA strain COL. The defect in PBP2 expression may partially contribute to the lower MIC; however, it was not the only factor contributing to the resistance mechanism, since the complementation of *pbp2* in *trans* did not significantly alter β-lactam resistance in *pbp4* mutants. The decrease in PBP2 combined with a loss in PBP4

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TABLE 1. Strains and plasmids

Strain or plasmid	Description	Reference or source
Strains		
S. aureus		
RN4220	MSSA derivative of 8325-4, acceptor of foreign DNA	35
MW2 (USA400)	CA-MRSA, wild-type strain	3
MW2 *pbp3*	Δ*pbp3* in-frame deletion mutant of parental strain MW2	This study
MW2 *pbp4*	Δ*pbp4* in-frame deletion mutant of parental strain MW2	This study
MW2 *pbp3/4*	Δ*pbp3* Δ*pbp44* in-frame double deletion mutant of parental strain MW2	This study
MW2 *pbp4*::*pbp4*	Δ*pbp4* complemented with *pbp4* with pMAD cycling	This study
MW2 *pbp4*$^+$	Wild-type complemented with pMAD cycling	This study
MW2 ex	CA-MRSA; wild-type strain cured of plasmid pMW2	This study
MW2-ex::pMW2	CA-MRSA; wild type cured of plasmid and complemented with pMW2	This study
MW2-ex *pbp3*	Δ*pbp3* in-frame deletion mutant of parental strain MW2 ex	This study
MW2-ex *pbp4*	Δ*pbp4* in-frame deletion mutant of parental strain MW2 ex	This study
MW2-ex *pbp3/4*	Δ*pbp3* Δ*pbp4* in-frame double deletion mutant of parental strain MW2 ex	This study
COL	HA-MRSA; wild-type strain	19
COL *pbp3*	Δ*pbp3* in-frame deletion mutant of parental strain COL	This study
COL *pbp4*	Δ*pbp4* in-frame deletion mutant of parental strain COL	This study
COL *pbp3/4*	Δ*pbp3* Δ*pbp4* in-frame double deletion mutant of parental strain COL	This study
USA300	CA-MRSA; wild-type strain	9
USA300 *pbp4*	Δ*pbp4* in-frame deletion mutant of parental strain USA300	This study
USA300 *pbp4*$^+$	Wild type complemented with pMAD cycling	This study
N315	HA-MRSA; wild-type strain	36
N315 *pbp4*	Δ*pbp4* in-frame deletion mutant of parental strain N315	This study
Mu50	HA-MRSA/VISA; wild-type strain	36
Mu50 *pbp4*	Δ*pbp4* in-frame deletion mutant of parental strain Mu50	This study
Newman *arlRS*	Δ*arlRS* in-frame deletion of methicillin-sensitive strain Newman	This study
E. coli strain XL-1 blue	General laboratory cloning strain	50
Plasmids		
pMAD	Allelic replacement vector to generate *S. aureus* mutant strains	2
pMAD-CM	pMAD containing the *cat* gene from pSK236 using NaeI	This study
pEPSA5	Ectopic expression vector (pEPSA5) for genes in *S. aureus*	12
pALC1484	Derivative of pSK236 containing recombinant gfp_{uvr} gene	29
pMAD MW2 *pbp3*−	pMAD containing ~0.8-kb up- and downstream MW2 fragments of *pbp3*	This study
pMAD MW2 *pbp4*−	pMAD containing ~0.8-kb up- and downstream MW2 fragments of *pbp4*	This study
pMAD MW2 *pbp4*+	pMAD containing the *pbp4* gene and ~0.8-kb up- and downstream from MW2	This study
pMAD COL *pbp3*−	pMAD containing ~0.8-kb up- and downstream COL fragments of *pbp3*	This study
pMAD COL *pbp4*−	pMAD containing ~0.8-kb up- and downstream COL fragments of *pbp4*	This study
pEPSA5::*pbp4*	pEPSA5 containing a 1.38-kb DNA fragment containing the *pbp4* coding region including its own ribosome binding site from MW2 at BamHI/XbaI	This study
pEPSA5::*pbp2*	pEPSA5 containing a 2.18-kb DNA fragment containing the *pbp2* coding region including its own ribosome binding site from MW2 at XmaI/BamHI	This study
pEPSA5::*mecA*	pEPSA5 containing a 2-kb DNA fragment containing the *mecA* coding region including its own ribosome binding site from MW2 at BamHI/XbaI	This study
pALC3711	pALC1484 with a 970-bp promoter fragment of *pbp2* (P1) fused with the gfp_{uvr} reporter gene at the EcoRI/XbaI sites	This study
pALC4677	pALC1484 with a 180-bp promoter fragment of *pbp2* (P2) fused with the gfp_{uvr} reporter gene at the EcoRI/XbaI sites	This study

might, however, explain the more significant loss in peptidoglycan cross-linking in CA-MRSA than in HA-MRSA. We further demonstrate that cefoxitin, a β-lactam that binds PBP4 irreversibly, renders CA-MRSA strains such as MW2 and USA300 as well as clinical isolates from skin and soft tissue infections sensitive again to oxacillin (MIC < 1 μg/ml). These findings have heightened the role of PBP4 in conferring methicillin resistance in CA-MRSA isolates. The combination of cefoxitin and oxacillin might constitute a valid therapeutic approach for treating CA-MRSA infections.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *Escherichia coli* strain XL-1 Blue was used in molecular cloning experiments. The wild-type and mutant *S. aureus* strains used in this study are listed in Table 1. Luria-Bertani medium (Becton Dickinson) was used for the culture of *E. coli*, while *S. aureus* was cultured in Mueller-Hinton broth (Becton Dickinson) supplemented with 25 μg/ml Ca^{2+}, 12.5 μg/ml Mg^{2+}, and 2% NaCl (cation-supplemented Mueller-Hinton broth [CSMHB]). When appropriate, antibiotics were added to the media at the following concentrations: ampicillin at 100 μg/ml for *E. coli* and chloramphenicol at 10 μg/ml and erythromycin at 2.5 μg/ml for *S. aureus*. Chloramphenicol was routinely used to maintain selection for pEPSA5- and pSK236-based plasmids (12). All antibiotics were obtained from Sigma.

Collection of clinical isolates and fast screening of CA-MRSA versus HA-MRSA strains. Two independent batches of clinical isolates were obtained from the Dartmouth Hitchcock Medical Center, Lebanon, NH. The first group included 200 blood MRSA isolates subcultured on 5% Columbia sheep blood agar (Remel), while the second group involved 30 MRSA isolates from complicated skin and soft tissue infections (CSSTI). All strains were grown overnight in CSMHB broth and streaked onto selective mannitol salt agar plates for confirmation. DNA extraction was performed on 500 μl of cultures. PCR was per-

formed on extracted DNAs with custom oligonucleotides (Integrated DNA Technology) specific for *mecA* (this study), *pvl* (38), and the three genes that we found to be specific for SCC*mec* type IV in CA-MRSA, MW0042, MW0043, and MW0047 (this study), without assigned functions. DNA amplification of *mecA* was used as a control to confirm that all strains were MRSA. DNAs from *S. epidermidis*, *E. coli*, and *Streptococcus pneumoniae* were used as negative controls to confirm the specificity of the oligonucleotides. After amplification for 30 cycles (5 min of denaturation at 95°C, 30 s of annealing at 54°C, and 1 min of extension at 72°C), PCR products were resolved by electrophoresis through 1.5% agarose gels stained with ethidium bromide. Given the number of clinical isolates tested, only a small random group of PCR products were subjected to DNA sequencing to confirm specificity. *S. aureus* strains COL, Mu50, N315, and MRSA252 all tested negative for MW0042, MW0043, and MW0047 and *pvl*, while USA300 and MW2 were positive controls for this PCR. The primer sequences for *mecA*, MW0042, MW0043, and MW0047 were as follows: mecA-UP (5′-GGTACTGC TATCCACCCTCAAA-3′), mecA-LOW (5′-TTACGACTTGTTGCATACCAT CA-3′), MW0042-UP (5′-ATTGGCAGAAATAAACAAAACG-3′), MW0042-LOW (5′-TCGTTTAATTTTTTCCCAAACTC-3′), MW0043-UP (5′-GTTTCA GTTGGTGTTGAAGATCC-3′), MW0043-LOW (5′-CCTATAATTTTCGAT AGATTCGTG-3′), MW0047-UP (5′-AGGCATATAAAGAAGCAGGAAAG-3′), and MW0047-LOW (5′-CACACTGTTTTCCTACGATATTTG-3′).

Susceptibility testing and synergy assay. MICs and minimum bactericidal concentrations (MBCs) were determined for each isolate and mutant in triplicate by microdilution techniques using an inoculum of 5×10^5 CFU/ml according to Clinical and Laboratory Standards Institute guidelines (6). For each mutant, three independent clones were tested. For MBC determinations, aliquots (5 μl) from clear wells were plated onto tryptic soy agar drug-free plates followed by incubation at 37°C for 24 and 48 h. MIC data were reported as median values from at least three independent experiments for each antibiotic. Strains containing pEPSA5-based plasmids were tested with and without xylose induction, but chloramphenicol was not added to avoid interference with β-lactam resistance evaluation. To assess the synergistic effect of cefoxitin plus oxacillin, we first determined the MIC of cefoxitin for both CA-RMSA and HA-MRSA strains; 0.25× MIC of cefoxitin was then used in combination with a twofold dilution of oxacillin to ascertain their synergistic effect. The correct inocula were confirmed by plating serial dilutions onto agar. The synergistic inhibitory activity of cefoxitin with oxacillin was scored at 24 and 48 h. Cefuroxime, a β-lactam with a very low affinity for PBP4, was also tested as a negative control in combination with twofold dilutions of oxacillin and was found to have no effect whatsoever on oxacillin MICs.

Population analysis. Antibiotic susceptibilities for MW2 were also determined by population analysis, as described previously (55). Briefly, the cells were grown overnight in CSMHB at 37°C, and four different dilutions of the bacterial culture (10^0, 10^{-2}, 10^{-4}, and 10^{-6}) were then plated onto control plates without antibiotic and onto plates containing a series of twofold dilutions of cefoxitin, oxacillin, or oxacillin combined to one-fourth the MIC of cefoxitin. The plates were incubated at 37°C for 48 h, and colonies were counted.

DNA and computational techniques. Plasmid DNA was isolated by standard techniques (Qiagen) from *E. coli* and from lysostaphin digestion of *S. aureus*. Chemically competent *E. coli* or electrocompetent *S. aureus* cells were used for transformation. New England Biolabs restriction endonucleases and ligases were used according to the manufacturer's recommendations. Iproof DNA polymerase from Bio-Rad Laboratories was used to generate all DNA fragments for deletions, promoter fusions, and ectopic expressions in pEPSA5. The fidelity of all DNA sequences generated by PCR was verified by fluorescently labeled dideoxynucleotide sequencing (Big Dye terminators; PE Applied Biosystems).

Construction of *S. aureus* mutants. All mutants were generated with an in-frame deletion of target genes by allelic replacement using the temperature-sensitive plasmid pMAD. Briefly, ~0.8-kb PCR products upstream and downstream of targeted sequences were generated and ligated by gene SOEing (26). The resulting ~1.6-kb product was digested, gel purified, cloned into pMAD using the same restrictions sites, and transformed into *E. coli*. Colony PCR was performed on *E. coli* transformants. Plasmids from positive clones were verified by digestion analysis and then used to transform *S. aureus* RN4220, selecting for erythromycin- or chloramphenicol-resistant and blue colonies at 30°C. Plasmid from RN4220 was sequenced and used to transform *S. aureus* strains MW2, COL, USA300, N315, and Mu50, for which the gene was to be deleted. The process of allelic replacement was described previously (2). For Mu50, N315, and USA300, which are erythromycin resistant, a modified pMAD vector was constructed (pMAD-CM) by cloning the chloramphenicol acetyltransferase (*cat194*) gene from pSK236 into the NaeI site of pMAD. All chromosomal deletions were verified by PCR and DNA sequencing. A minimum of three independent clones for each mutant were generated in every genomic background analyzed and studied further. The resulting deletion strains were devoid of the entire open reading frame (ORF) for both *pbp3* and *pbp4*. The same pMAD system was also utilized to reinsert the *pbp4* ORF into the MW2 Δ*pbp4* and Δ*pbp3* Δ*pbp34* mutants and the USA300 Δ*pbp4* mutants for complementation. The sequences of DNA primers used in this study are available from the authors upon request.

Curing of plasmid, loss of cadmium resistance, β-lactamase production, and *mecA* repression. The loss of plasmid pMW2 in strain MW2 and, therefore, the loss of resistance to cadmium and β-lactamase production were investigated by passaging MW2 at 44°C and screening single colonies by a replica plate method. Colonies that were sensitive to cadmium and tested positive for the loss of β-lactamase production as determined by nitrocefin assay were tested for plasmid loss by agarose gel electrophoresis and PCR with oligonucleotides specific for *blaIRZ*. The expression of *mecA* in the strain cured of the plasmid (MW2 ex) was evaluated by Northern blot analysis.

Isolation of RNA and Northern blot hybridization. Cultures of *S. aureus* grown overnight were diluted 1:100 in 40 ml of CSMHB broth and grown with shaking to exponential phase (A_{650} of 0.7) in 200-ml flasks. At an optical density (OD) of 0.7 (Spectronic 20 using 18-mm borosilicate glass tubes), total RNA was extracted from 10 ml of culture, while the remaining 30 ml was divided evenly into three glass tubes, one with no antibiotic, the second with 10× MIC of oxacillin, and the third with 10× MIC of vancomycin. After growing for an additional 60 min, RNAs from all samples were extracted by using a Trizol-glass bead method as described previously (40). The concentration of total RNA was determined by measuring the absorbance at 260 nm. Ten micrograms each of total RNA was analyzed by Northern blotting as described previously (6). Each DNA probe (350 bp) was generated by PCR from chromosomal templates. For the detection of specific transcripts, gel-purified DNA probes were radiolabeled with [α-^{32}P]dCTP by using the random-primed DNA labeling kit (Roche Diagnostics GmbH) and hybridized under aqueous-phase conditions at 65°C. The blots were subsequently washed, and bands were visualized by autoradiography.

Transcriptional fusion studies of PBP2 promoters linked to the GFP$_{uvr}$ reporter gene. To confirm the effect of the *pbp4* deletion on *pbp2*, we cloned both *pbp2* promoters in pALC1484, a derivative of pSK236 containing the promoterless *gfp$_{uvr}$* to generate transcriptional fusions. Clustal W analysis revealed that the promoter sequences from HA-MRSA strain COL and CA-MRSA strain MW2 are 100% identical. Restriction analysis and DNA sequencing confirmed the orientation and authenticity of the promoter fragments upstream of the reporter gene. Recombinant plasmids were introduced into *S. aureus* RN4220, purified, and electroporated into wild-type MW2, COL, and their isogenic *pbp* mutants. Cultures grown overnight were diluted 1:100 in CSMHB with chloramphenicol and grown to an OD at 650 nm (OD_{650}) of 0.7; bacterial cultures were then exposed to no antibiotic, oxacillin, or vancomycin at 10× MIC for 60 min. Aliquots of 200 μl were transferred before antibiotic challenge and every hour thereafter into microtiter wells to assay for cell density (OD_{650}) and fluorescence in an FL600 fluorescence reader (BioTek Instruments). Promoter activation was plotted as the mean fluorescence/OD_{650} ratio, using the average values from triplicate readings from three clones per strain.

Ectopic expression of genes in *S. aureus*. To complement the mutant strains, we also utilized expression plasmid pEPSA5, which can be induced for expression with xylose (1%) or repressed by glucose (1%) (11). For pEPSA5-mediated expression, genes were amplified by PCR, and products were digested, gel purified, ligated into pEPSA5, and transformed into *E. coli* XL-1 Blue. Plasmids from positive clones in *E. coli* were verified by digestion analysis and then used to transform *S. aureus* RN4220. The plasmids were then introduced into MW2, USA300, and their isogenic *pbp4* mutants as described above. To confirm the correct induction with 1% xylose with recombinant pEPSA5, reverse transcription-PCR of the specific transcript was performed on RNAs before and after xylose induction. Briefly, total RNA was extracted, resuspended in diethyl pyrocarbonate water, and treated with Turbo DNase (Ambion). cDNA was generated from 1 μg of cellular RNA using the Transcriptor first-strand cDNA synthesis kit (Roche). Regular PCR was then performed on cDNAs using oligonucleotides specific for each gene. Nonetheless, the basal level of expression was always observed without adding exogenous xylose to culture media.

Triton X-100-induced autolysis assays in static cultures. The autolysis assay was performed as described previously (28). Briefly, strains grown overnight in tryptic soy broth were diluted and grown to mid-logarithmic phase (A_{650} of 0.7). Cells were washed twice in cold sterile distilled water and resuspended in 10 ml of 0.05 M Tris-HCl (pH 7.2) containing 0.05% Triton X-100 at 30°C, and the A_{600} was measured every 30 min. Data are expressed as the percent loss of A_{600} at the indicated times compared to the zero time point. Each data point represents the mean and standard deviation from three independent experiments.

Effect of Triton X-100 on growing cells. The effect of Triton X-100 was assayed on actively dividing cells as described previously (28). Briefly, cultures grown

overnight were diluted to an OD_{650} of 0.1 in CSMHB with different concentrations of Triton X-100. Cells were incubated at 37°C with shaking, and ODs were recorded hourly for 7 to 8 h. Each data point represents the mean and standard deviation from three independent experiments.

Zymogram analysis. Zymogram analysis was conducted to detect alterations in autolysin activity as previously described, with minor alterations (28). Heat-killed RN4220 cells were incorporated into an 8% sodium dodecyl sulfate (SDS)-polyacrylamide gel at 10 mg/ml wet weight. Autolytic enzymes were extracted from 10 ml of culture grown to an A_{650} of 0.7 using 100 μl of 4% SDS. Equivalent amounts of proteins were separated on an SDS-polyacrylamide gel. Resolved proteins were allowed to renature overnight in water and incubated with 0.1% methylene blue to visualize clear bands, representing an area of RN4220 cell lysis. The assay was repeated three times, with a representative experiment shown.

Electron microscopy. Cultures grown overnight were reinoculated in fresh CSMHB and grown to an OD_{650} of 0.7. One 1-ml aliquot was harvested by low-speed centrifugation. Oxacillin was then added to the other two specimens to achieve a final concentration of 1× MIC and 10× MIC, with cells growing at 37°C for an additional 60 min, and then harvested. All specimens described above were then fixed with 2% glutaraldehyde–1% paraformaldehyde and post-fixed with OsO_4. Ultrathin sections were stained with 2% methanolic uranyl acetate and lead citrate and examined with a Jeol JEM 1010 electron microscope.

Analysis of peptidoglycan. Peptidoglycan and muropeptide preparation and consequent high-performance liquid chromatography (HPLC) separation were performed as described previously (8). Briefly, cell wall extracts were purified by shaking bacteria with glass beads, followed by enzymatic digestion of DNA, RNA, and proteins. Peptidoglycan was then obtained by treating cell wall extracts with hydrofluoric acid. Muropeptides obtained by digesting peptidoglycan with mutanolysin (Sigma) were separated by HPLC and detected by measuring the absorption at 206 nm. Quantification was made by measuring the area of each individual peak and expressing it as a percentage of the total area of all peaks. Peaks of interest were desalted and analyzed by matrix-assisted laser desorption ionization mass spectrometry using yano-4-hydroxycinnamic acid as the matrix-assisted laser desorption ionization matrix at the Mass Spectrometry Service at the ITQB, Lisbon, Portugal.

TABLE 2. Oxacillin, nafcillin, and cefoxitin MICs for wild-type and mutant strains[a]

Strain	PFGE serotype	MIC (μg/ml)		
		OXA	NAF	FOX
MW2 (USA400) (wild type)	USA400	64	16	64
MW2 Δ*pbp3*	USA400	64	16	64
MW2 Δ*pbp4*[c]	USA400	**4**	**1**	64
MW2 Δ*pbp3*/*4*[c]	USA400	**4**	**1**	64
MW2 Δ*pbp4*::*pbp4*	USA400	64	16	64
MW2 ex[b]	USA400	128	16	16
MW2 ex Δ*pbp3*	USA400	128	16	32
MW2 ex Δ*pbp4*[c]	USA400	**2**	**1**	16
MW2 ex Δ*pbp3*/*4*[c]	USA400	**1**	**1**	32
USA300 (wild type)	USA300	64	16	64
USA300 Δ*pbp*4	USA300	**4**	**1**	64
USA300 Δ*pbp4*::*pbp4*[c]	USA300	64	16	64
COL wild-type	USA500	256	64	128
COL Δ*pbp*3	USA500	256	64	128
COL Δ*pbp*4	USA500	256	64	128
COL Δ*pbp3*/*4*	USA500	256	64	128
N315 (wild type)	USA100	256	64	64
N315 Δ*pbp*4	USA100	128	64	64
Mu50 (wild type)	USA100	256	256	256
Mu50Δ*pbp*4	USA100	256	256	256

[a] Determinations of MICs of oxacillin, nafcillin, and cefoxitin for parental and isogenic *pbp* mutants were performed according to CLSI (formerly NCCLS) protocols. Bacteria, grown to an OD_{650} of 1, were added to CSMHB to a final concentration of 5×10^5 CFU/ml.

[b] MW2 ex, MW2 cured of plasmid pMW2.

[c] *pbp4* is essential for β-lactam resistance in CA-MRSA strains MW2 and USA300 but not in HA-MRSA COL, N315, and Mu50 (MRSA/VISA) (as shown bolded and highlighted).

RESULTS

Construction and characterization of PBP4 mutants. The role of PBPs and their contribution to β-lactam resistance in CA-MRSA have never been investigated. For this purpose, we deleted the ORFs encoding PBP3 (MW1504) and PBP4 (MW0604), both of which are transcribed monocistronically and reside at different parts of the chromosome in HA-MRSA strain COL and CA-MRSA strain MW2 (USA400). The PBP4 gene was also deleted in the hospital-acquired strains Mu50 and N315 and community-acquired strain USA300. We also tried but failed to obtain viable clones for ΔPBP1 and ΔPBP2. The PBP4 gene shares a 400-nucleotide intercistronic region with the divergently transcribed *abcA* (MW0605), encoding an ATP binding cassette transporter that has been shown to be involved in cell wall metabolism and division (10). Accordingly, we assessed and confirmed that our *pbp4* deletion did not lead to a polar effect on both the upstream (MW0603 [teichoic acid biosynthesis protein D {*tagD*}]) and the downstream (*abcA*) genes by Northern blot analysis. A minimum of three independent clones were analyzed for each mutant. Hypersensitivity to oxacillin and nafcillin was first determined by assessing the MICs for parents and isogenic *pbp* mutants (Table 2). Oxacillin and nafcillin resistances were not altered in *pbp3*, *pbp4*, or double mutants of COL, in concordance with previously reported results (30, 48). Similarly, the loss of PBP4 in HA-MRSA strains N315 and Mu50 resulted in a minimal or no decrease in MICs of both oxacillin and nafcillin. Remarkably, a loss of *pbp4* in MW2 and USA300 resulted in a 16-fold reduction in oxacillin and nafcillin MICs (from 64 to 4 μg/ml and from 16 to 1 μg/ml, respectively), while a deletion in *pbp3* had no effect.

Effect of *pbp4* deletion on *pbp2* and *pbp2A* expression. The ability of *S. aureus* to survive in the presence of β-lactams relies upon its ability to express PBP2A, the alternative PBP encoded by *mecA*. The transglycosylase domain of PBP2 is a second crucial determinant for β-lactam resistance in MRSA strains (47). Recent data have shown that PBP2 affects the expression of *mecA* and also functions cooperatively with the transpeptidase activity of PBP4 (15, 37). To assess whether the loss of resistance in the *pbp4* mutants of CA-MRSA was due to defective *pbp2* or *mecA* expression, we performed Northern blots and showed that a loss of *pbp4* diminished the induced expression of *pbp2* upon exposure to both cell wall-active antibiotics oxacillin and vancomycin in CA-MRSA MW2 (Fig. 1A) but not in HA-MRSA COL (Fig. 1C). In contrast, the transcript level of *mecA* was not altered in the *pbp4* mutants of both MW2 and COL (Fig. 1B to D). The defect in the induction of *pbp2* expression with oxacillin in the *pbp4* mutant of MW2 was restored to wild-type levels upon complementation (Fig. 1E), while the empty vector had no effect. To confirm the effect of the *pbp4* deletion on *pbp2* expression, the P1 (2.9-kb transcript) and P2 (2.2-kb transcript) promoters of *pbp2* were fused to a promoterless gfp_{uvr} reporter gene in shuttle plasmid pALC1484. Both P1 and P2 promoters were induced by oxacillin at 10× MIC in MW2 but not in the *pbp4* or *pbp4 pbp3* mutant, confirming the Northern blot data (Fig. 2A to D). The differences in green fluorescent protein (GFP) values were not due simply to a difference in growth between the wild-type and the mutant strains because the ODs after 4 h of oxacillin

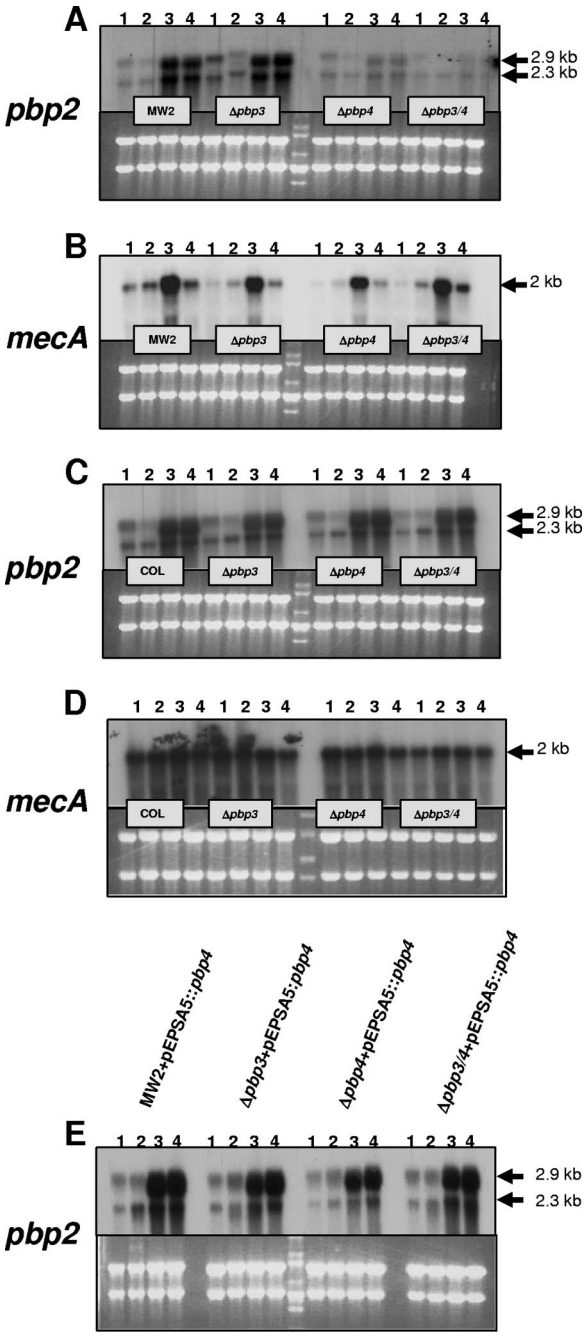


FIG. 1. Loss of *pbp4* affects the expression of *pbp2* in cells challenged with oxacillin in CA-MRSA strain MW2 but not in HA-MRSA strain COL. Shown is Northern blot analysis of *pbp2* and *mecA* expression (A and B, MW2; C and D, COL) in cultures with no antibiotic, oxacillin, or vancomycin at 10× MIC for 1 h. Blots were hybridized with a DNA probe specific for *pbp2* (A and C) or *mecA* (B and D) radiolabeled with [α-^{32}P]dCTP. (Bottom) Ethidium bromide-stained rRNAs indicating equivalent amounts of RNA in each sample. Lane 1, RNA from cells at an OD_{650} of 0.7 before antibiotic induction; lanes 2 to 4, RNA from cells at 1 h with no antibiotic, oxacillin, and vancomycin at 10× MIC, respectively. (E) Northern blot analysis of *pbp2* expression in MW2 and its isogenic mutants carrying pPEPSA5::*pbp4*. Complementation of PBP4 in *trans* restored *pbp2* transcripts to wild-type levels, while the empty vector had no effect (data not shown). Lane 1, RNA from cells at 0.7 OD units before antibiotic induction; lanes 2 to 4, RNA from cells at 1 h with no antibiotic, oxacillin, and vancomycin at 10× MIC, respectively.

induction were virtually identical among all strains. Additionally, differences in GFP values were still apparent even after overnight growth, thus confirming that the disparity was due to a transcriptional defect instead of a growth defect. In contrast, differences in the induction of *pbp2* after antibiotic challenge were still apparent in *pbp4* and *pb4 pbp3* mutants of strain COL (Fig. 2E to H), wherein both P1 and P2 promoters were equally upregulated in the presence of oxacillin and vancomycin in both wild-type and mutant strains.

Complementation studies. To exclude chromosomal rearrangement or an ectopic point mutation as the cause for the loss of resistance, plasmid pMAD was used to reintroduce *pbp4* into the chromosome of Δ*pbp4* and Δ*pbp3*Δ *pbp4* mutants of MW2. Both chromosomally complemented strains MW2 Δ*pbp4*::*pbp4* and USA300 Δ*pbp4*::*pbp4* regained wild-type levels of oxacillin resistance (Table 2). Similar results were obtained when the ORF encoding PBP4 was cloned in the xylose-inducible system pEPSA5, while the empty vector had no effect (data not shown). However, the expression of PBP2 or PBP2A in pEPSA5 did not alter the MIC in both *pbp4* and *pbp3 pbp4* mutants (data not shown). Notably, the reduction in oxacillin resistance in the *pbp4* mutants of MW2 and USA300 was not fully attributable to *pbp2* because the expression of *pbp2* from the inducible plasmid pEPSA5 did not restore oxacillin resistance in the *pbp4* mutant, with the MIC still at 4 μg/ml. To further exclude possible polar effects due to genetic manipulation of the ORFs encoding PBP3 and PBP4, transcription profiles of genes upstream and downstream of both ORFs were examined in MW2, revealing no significant alterations between the wild type and the mutants (data not shown).

MecA regulation-expression and β-lactam resistance. The hospital-acquired strain COL and the community-acquired strain MW2 have substantially different regulations of *mecA*. Both strains lack the gene encoding *mecI*, a repressor of *mecA* expression. However MW2 carries the *blaIRZ* operon on plasmid pMW2, which codes for the penicillinase-regulatory gene *blaI*, the membrane sensor *blaR*, and the penicillinase gene *blaZ*; *blaI* was previously found to regulate the expression of *mecA* in *trans* (41). To ascertain the role of plasmid pMW2 in the regulation of *mecA* in MW2, we cured the plasmid to generate the derivative strain MW2 ex. This strain resulted in the constitutive expression of *mecA*, similar to what has been found with COL (Fig. 3). In the complemented strain obtained by reintroducing plasmid pMW2 into MW2 ex, the expression of *mecA* was again repressed unless oxacillin was added to induce expression. The loss of *pbp4* in MW2 ex, with or without a concomitant loss of *pbp3*, led to a more substantial decrease in oxacillin resistance than in MW2, with a 64-fold reduction in the oxacillin MIC for the *pbp4* mutant (from 128 to 2 μg/ml in MW2 ex versus 64 to 4 μg/ml in MW2) and a 128-fold reduction for the *pbp3 pbp4* double mutants (from 128 to 1 μg/ml in MW2 ex versus 64 to 4 μg/ml in MW2), respectively (Table 2).

Effect of *pbp4* deletion on genes that regulates autolysis and murein hydrolase activity. To assess whether hypersensitivity to β-lactams in *pbp4* mutants of MW2 was due to an effect on regulatory autolytic genes and/or increased murein hydrolase activity, Northern blots were conducted with DNA probes specific for the following genes: *sarA*, *sarR*, *sarT*, *sarS*, *mgrA*, *atl*, *lytM*, *lytN*, *lysR*, *lytSR*, *cidABC*, and *lrgAB*. Besides a minor difference in *sarA* transcription in all *pbp* mutants, all other

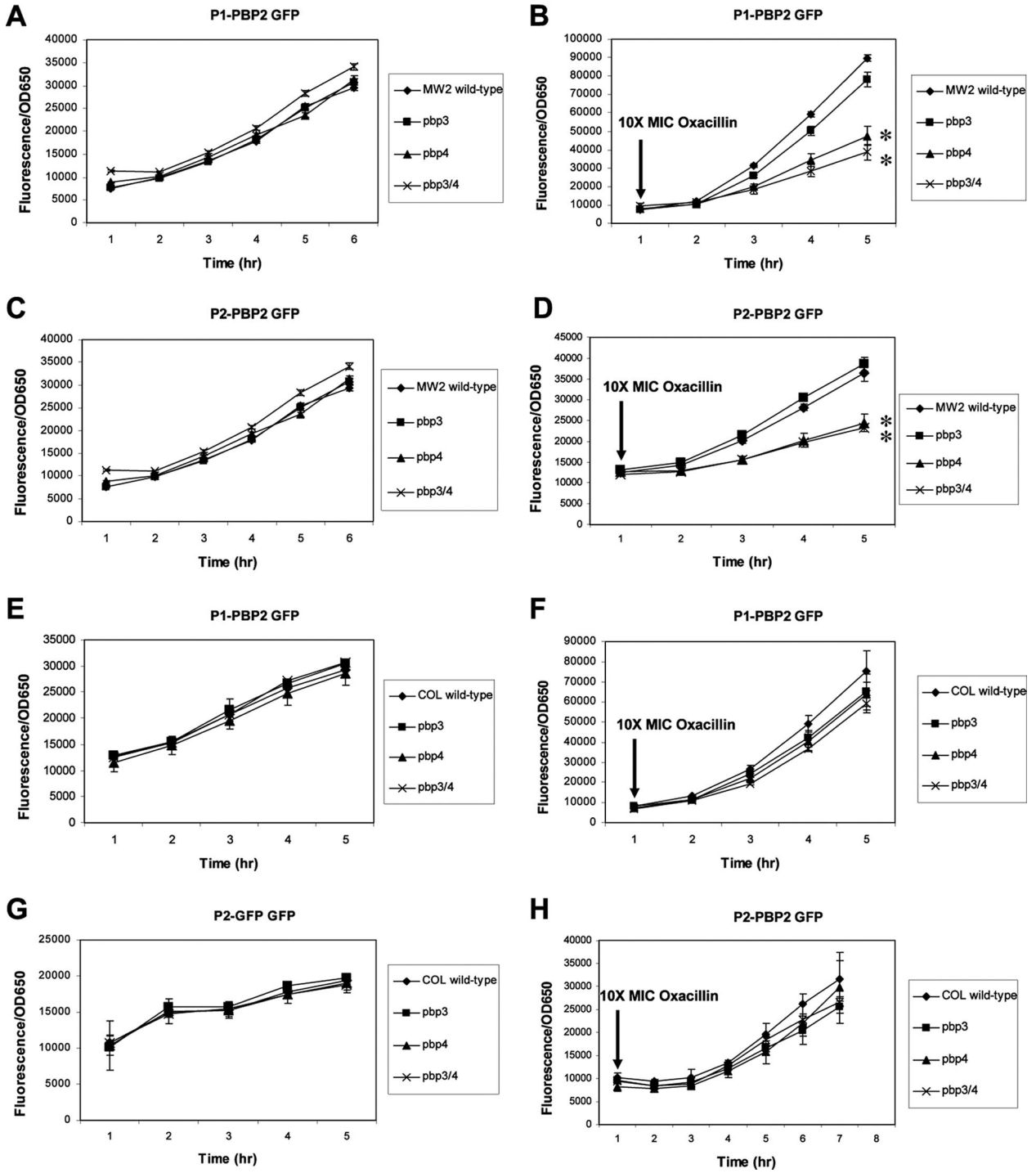


FIG. 2. Expression of GFP_{uvr} driven by *pbp2*-P1 (A and B, MW2; E and F, COL) and *pbp2*-P2 (C and D, MW2; G and H, COL) promoters in uninduced (A, C, E, and G) and induced (B, D, F, H) cultures with 10× MIC of oxacillin. Cultures grown overnight were reinoculated in CSMHB and grown to mid-log phase followed by the addition of no antibiotic or 10× MIC of oxacillin. Promoter activity was plotted as the mean fluorescence/OD_{650} from three clones in triplicates. The experiments were repeated three times, with one set shown. The "*" indicates statistical significance of the indicated strain to MW2 at 3 to 6 h after antibiotic challenge by the paired Student's *t* test ($P < 0.001$).

transcript levels tested were similar between wild types and their isogenic mutant strains (data not shown). Zymogram analysis also did not reveal enhanced autolysis, with similar murein hydrolase activities between MW2 and all *pbp* mutants (Fig. 4A and B). We also checked the effect of the nonionic detergent Triton X-100 on static and actively growing cells. However, no differences in ODs were observed between the wild type and the mutants in static cultures exposed to 0.05%

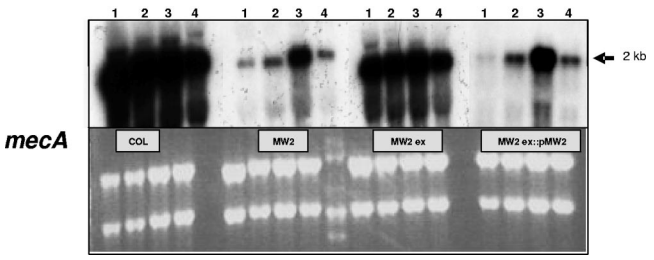


FIG. 3. Northern blot analysis of *mecA* expression in CA-MRSA strain MW2 (USA400) and its isogenic strain MW2 ex, cured of plasmid pMW2. The expression of *mecA* was assessed in cultures with no antibiotic, oxacillin, or vancomycin at 10× MIC for 1 h. Blots were hybridized with a DNA probe specific for *mecA* radiolabeled with [α-^{32}P]dCTP. (Bottom) Ethidium bromide-stained rRNAs indicating equivalent loading of RNA in each sample. Lane 1 corresponds to RNA from cells at 0.7 OD units before induction with oxacillin; lanes 2 to 4 correspond to RNA from cells at 1 h with no antibiotic induction, oxacillin at 10× MIC, and vancomycin at 10× MIC, respectively.

Triton X-100 at 30°C (Fig. 4C), thus suggesting that the amounts of autolysins in the mutated strain are equal to those associated with the wild type, while the two-component system *arlRS* mutant in the Newman background, used as a positive control, did reveal increased autolysis. In growing cultures at 37°C with Triton X-100, both *pbp4* and *pbp4 pbp3* mutants showed a reduced rate of the rise in the OD compared with that of the wild type or complemented *pbp4* mutant. This discrepancy in the rise of OD between static and actively growing cultures is more consistent with a defect in cell wall biosynthesis than with augmented autolytic activity (Fig. 4D).

***pbp4* deletion affects the level of peptidoglycan cross-linking.** Previous studies linked the loss of PBP4 to a reduction in peptidoglycan cross-linking in methicillin-sensitive *S. aureus*, MRSA COL, and glycopeptide-resistant *S. aureus* strains (53). HPLC elution profiles of muropeptide species released by muramidase digestion of peptidoglycan from MW2 and its *pbp* mutants (Fig. 5) divulged an increase in monomers, dimers, trimers, and tetramers and a concomitant reduction in the proportion of highly cross-linked muropeptides (>tetramer) in *pbp4* mutants but not in *pbp3* mutants.

Effect of loss of *pbp4* on cell morphology and septum formation. Electron microscopy revealed no major differences between MW2 and *pbp4* mutants without β-lactam challenge, while the exposure of cells to oxacillin led to incomplete septum formation, irregularly placed septa, and rough cell wall surfaces in parents and mutant strains alike (data not shown).

Synergistic effect of cefoxitin and oxacillin in CA-MRSA strains. Although PBP4 binds poorly to many β-lactams, it

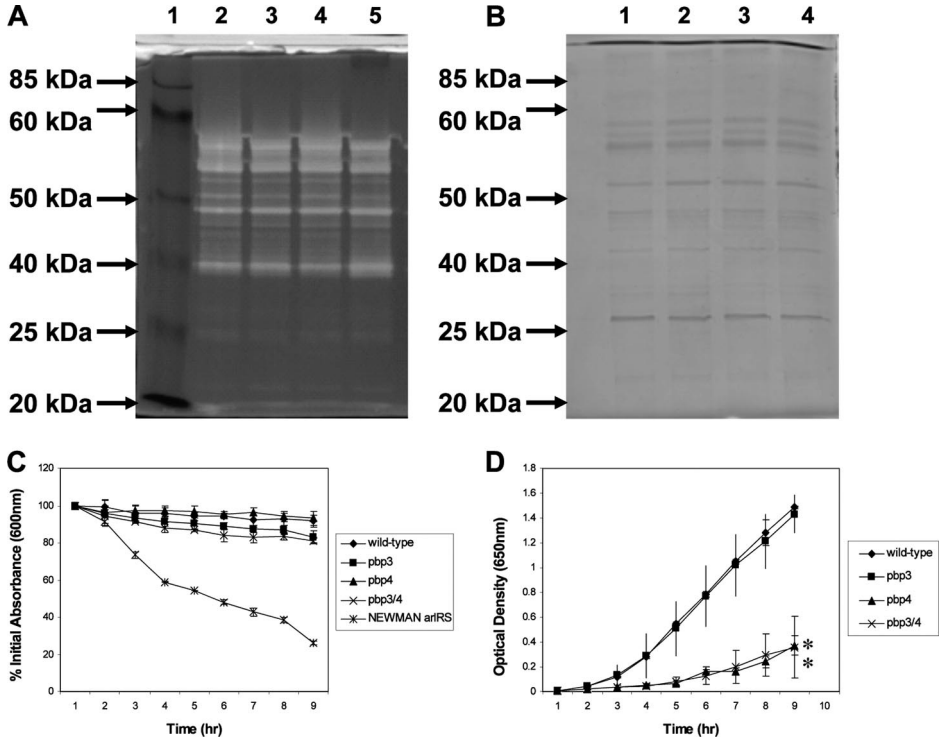
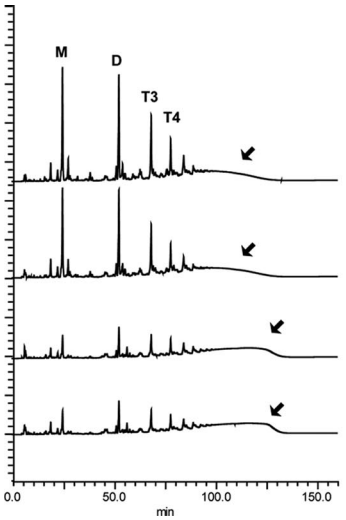


FIG. 4. Zymogram analysis and autolysis assays of *pbp4* mutants with 1% Triton X-100. (A) Zymogram analysis of cell extracts from *S. aureus* MW2 and its isogenic *pbp* mutants. Lanes: 1, molecular size markers; 2, MW2; 3, Δ*pbp3*; 4, Δ*pbp4*; 5, Δ*pbp3* Δ*pbp4*. Equivalent amounts of cell extracts were separated on a 12% SDS-polyacrylamide gel containing heat-killed *S. aureus* RN4220 cells, treated with 1% Triton X-100, and stained with 1% methylene blue. Areas of murein hydrolase activity are indicated by clear zones. (B) Coomassie-stained gel of A. (C) Triton X-100-induced autolysis assay under static conditions. After adding Triton X-100 (0.05%), the autolysis of mid-exponential-phase cultures was determined at 30°C without shaking by serial OD measurements. The *arlRS* mutant of strain Newman was used as a positive control. (D) Effect of Triton X-100 on growing cells of MW2 and its isogenic mutants. Cultures grown overnight, diluted to an OD_{650} of 0.1 in CSMHB with 0.05% Triton X-100, were grown at 37°C with shaking. The "*" indicates statistical significance of *pbp3* and *pbp3 pbp4* mutants to MW2 at 4- to 9-h time points by the paired Student's *t* test ($P < 0.001$).



	Monomer (M)	Dimer (D)	Trimer (T3)	Tetramer (T4)	> Tetramer (> T4)
MW2Δ*pbp3*Δ*pbp4*	11,3	10,1	8,1	5,0	65,6
MW2Δ*pbp4*	10,5	9,3	7,4	4,7	68,2
MW2Δ*pbp3*	2,9	3,3	4,0	2,8	87,1
MW2	2,8	3,3	3,1	2,7	88,0

FIG. 5. Effect of deleting *pbp3* and *pbp4* on MW2 peptidoglycan composition. Peptidoglycan was prepared from exponentially grown bacteria in tryptic soy broth medium, as described previously (15). The muropeptide composition of peptidoglycan was analyzed by HPLC. The identity of each peak was assigned based on the specific retention time in comparison to previously identified peaks of strain COL, with arrows pointing to highly cross-linked muropeptides present in MW2 and its isogenic *pbp3* mutant but reduced in *pbp4* and *pbp3 pbp4* mutants. Quantification of the area of eluted peaks was carried out using Shimadzu LC solution software, with the data presented as a percentage of the total identified peaks.

does bind cefoxitin with high affinity (46). We thus tested the synergistic effect of cefoxitin (0.25× MIC) in combination with oxacillin by determining the MIC of oxacillin with CA-MRSA and HA-MRSA strains (Table 3). Oxacillin resistance was minimally altered by the presence of cefoxitin in HA-MRSAs and VISA strain Mu50. Remarkably, a synergistic inhibitory effect of cefoxitin with oxacillin was found with MW2 and USA300, with both strains being unable to grow at <1 μg/ml of oxacillin at both the 24- and 48-h time points. We also tested the MBC of oxacillin, cefoxitin, and oxacillin combined to one-fourth the MIC of cefoxitin in both CA-MRSA and HA-MRSA strains (Table 4). The MBC of oxacillin for both MW2 and USA300 was 256 μg/ml, while that of cefoxitin was >256 μg/ml. However, the MBC of oxacillin with one-fourth the MIC of cefoxitin was <1 μg/ml for both MW2 and USA300 (Table 4). Population analysis of susceptibility to oxacillin, cefoxitin, and oxacillin combined to one-fourth the MIC of cefoxitin to detect trends and the potential for developing resistance was also evaluated (Fig. 6). Our population analysis continued to demonstrate the synergistic inhibitory effect of cefoxitin and oxacillin on CA-MRSA strain MW2, with the MIC of oxacillin dropping to a level that is still clinically achievable, from 128 to 0.5 μg/ml.

Synergistic effect of cefoxitin and oxacillin in clinical isolates and CA-MRSA strains. To assess the efficacy of the synergistic combination of cefoxitin and oxacillin on other CA-MRSA strains, we screened 200 MRSA isolates, blinded with respect to origin, from Dartmouth-Hitchcock Medical Center, Lebanon, NH. Analysis of the type IV SCC*mec* element, virtually identical between MW2 and USA300, allowed us to identify three genes conserved in the CA-MRSA background but not in other known MRSA genomes. PCR of chromosomal DNAs with oligonucleotides specific for *mecA*, *pvl* (38), and the three specific genes for CA-MRSAs (MW0042, MW0043,

TABLE 3. Oxacillin and cefoxitin MICs for wild-type CA-MRSA and HA-MRSA isolates in combination with one-fourth the MIC of cefoxitin[a]

Isolate	No. of clones tested	MIC (μg/ml)				
		OXA 48 h	FOX 48 h	OXA + 1/4 MIC FOX 24 h	OXA + 1/4 MIC FOX 48 h	OXA + 1/4 MIC CXM 48 h
MW2 (USA400) (CA-MRSA)	3	64	64	<1	<1	64
USA300 (CA-MRSA)	3	64	64	<1	<1	64
COL (HA-MRSA)	3	256	128	32	64	NA
MRSA252 (HA-MRSA)	3	256	128	64	128	NA
Mu50 (HA-MRSA/VISA)	3	256	256	128	256	NA
CA-MRSA strains	30	64[b]	64[b]	<1	<1	NA
CA-MRSA from CSSTI	30[c]	64[b]	64[b]	<1	<1	NA
HA-MRSA strains	30	256[b]	128[b]	32[b]	64[b]	NA

[a] Cefoxitin exerts a synergistic effect with oxacillin on CA-MRSA strains, while cefuroxime, a β-lactam with very low affinity for PBP4, did not yield a similar outcome. MICs were determined with cultures grown overnight diluted in CSMHB with 0.25× MIC of cefoxitin in 96 microtiter wells to achieve a final concentration of 5×10^5 CFU/ml. All serial dilutions were plated onto antibiotic-free agar to confirm the inocula. Growth was checked after 24 and 48 h. NA, not applicable.

[b] MIC data for all clinical isolates were reported as median values from at least three independent experiments.

[c] Two CSSTI isolates showed MICs for this combination higher than those of the rest of the tested strains (MIC oxacillin was 4 μg/ml compared to 1 μg/ml, still several times below the maximum achievable concentration in vivo).

TABLE 4. MBCs of oxacillin, cefoxitin, and oxacillin in combination with one-fourth the MIC of cefoxitin for wild-type CA-MRSA and HA-MRSA strains[a]

Strain	No. of clones tested	MBC[b] μg/ml		
		OXA 48 h	FOX 48 h	OXA + 1/4 MIC FOX 48 h
MW2 (USA400) (CA-MRSA)	3	256	>256	<1
USA300 (CA-MRSA)	3	256	>256	<1
COL (HA-MRSA)	3	>256	>256	>256
MRSA252 (HA-MRSA)	3	>256	>256	>256
Mu50 (HA-MRSA/VISA)	3	>256	>256	>256

[a] MBC was assessed by plating the broth from the MIC wells and from those wells above the MIC for each strain onto appropriate drug-free growth media after 48 h and incubated at 37°C for 24 h. OXA, oxacillin; FOX, cefoxitin.
[b] MIC data for all clinical isolates were reported as median values from at least three independent experiments.

and MW0047) identified 30 isolates that were positive for these genes. All 30 isolates exhibited the synergistic inhibitory effect of cefoxitin and oxacillin, with 27 strains (90%) being unable to grow at <1 μg/ml of oxacillin after 24 and/or 48 h, and the remaining three grew at 1, 4, and 8 μg/ml of oxacillin with 0.25× MIC of cefoxitin after 48 h (Table 3). The HA-MRSA isolates that tested negative for the above-mentioned genes (but positive for *mecA*) grew in oxacillin concentrations ranging from 4 to 128 μg/ml (median values at 32 and 64 μg/ml at 24 and 48 h, respectively) in the presence of cefoxitin. The code was subsequently broken, confirming that all 30 isolates, designated by us as being CA-MRSA isolates, originated from patients with primary skin infections and annotated by the hospital as being CA-MRSA, while the other isolates were blood isolates designated HA-MRSA isolates. We further tested this synergistic inhibitory activity on 30 MRSA isolates from CSSTI, with CA-MRSA being the most common etiological agent. The MICs of cefoxitin for all strains were first obtained, and one-fourth of the median value was used to test the synergistic inhibitory activity with oxacillin. The MICs of oxacillin for 28 of them were <1 μg/ml when one-fourth the MIC of cefoxitin was added. Two isolates presented slightly higher values, with an MIC of 4 μg/ml, which we found to be attributable to a higher level of resistance to cefoxitin.

DISCUSSION

CA-MRSA isolates s were initially associated with lethal infections in children in the Midwest (21). CA-MRSA strains have since become the most frequent cause of skin and soft tissue infections in emergency rooms in the United States, increasing in frequency from 29% in 2001 to 2002 to 64% in 2003 to 2004 (32, 43, 44). However, the epidemiology of MRSA disease is changing, with the pulsed-field type USA300 now being the most common strain in the community but also emerging as a major cause of nosocomial bloodstream and prosthetic joint infections (33, 52). These epidemiological data underline the need to study the molecular mechanisms of virulence and drug resistance in this strain. PBP4, although not essential for viability, has been associated with low-level resistance to vancomycin and β-lactam antibiotics in HA-MRSA strain COL (11, 22, 24, 30, 45, 53). However, a mutation in *pbp4*, but not *pbp3*, in MW2 and USA300 strains has led to a significant decrease in high-level resistance to the penicillinase-stable antibiotics oxacillin and nafcillin, but this alteration in resistance was not observed in *pbp3*, *pbp4*, or double mutants of HA-MRSA strain COL. More specifically, there was a 16-fold reduction in oxacillin and nafcillin MICs (from 64 to 4 μg/ml and from 16 to 1 μg/ml, respectively) in *pbp4* mutants of MW2 and USA300, while a *pbp3* deletion had no effect. Remarkably, a *pbp4* mutation in HA-MRSA strain N315 and VISA strain Mu50 also did not result in decreased MICs of oxacillin.

The major contribution of PBP4 to resistance in CA-MRSA strains was validated by our findings that *mecA* expression remained unchanged in the *pbp4* mutants. The overexpression of *mecA* in inducible plasmid pEPSA5 did not complement β-lactam resistance in either the *pbp4* or the *pbp3 pbp4* mutant, with their MICs of oxacillin being unchanged. The loss of PBP4 in MW2 ex, an MW2 strain lacking plasmid pMW2 and constitutively expressing *mecA* in a manner similar to that of COL, was also found to have decreased oxacillin resistance (Δ*pbp4* MIC of 2 μg/ml versus 128 μg/ml in MW2 ex), more so

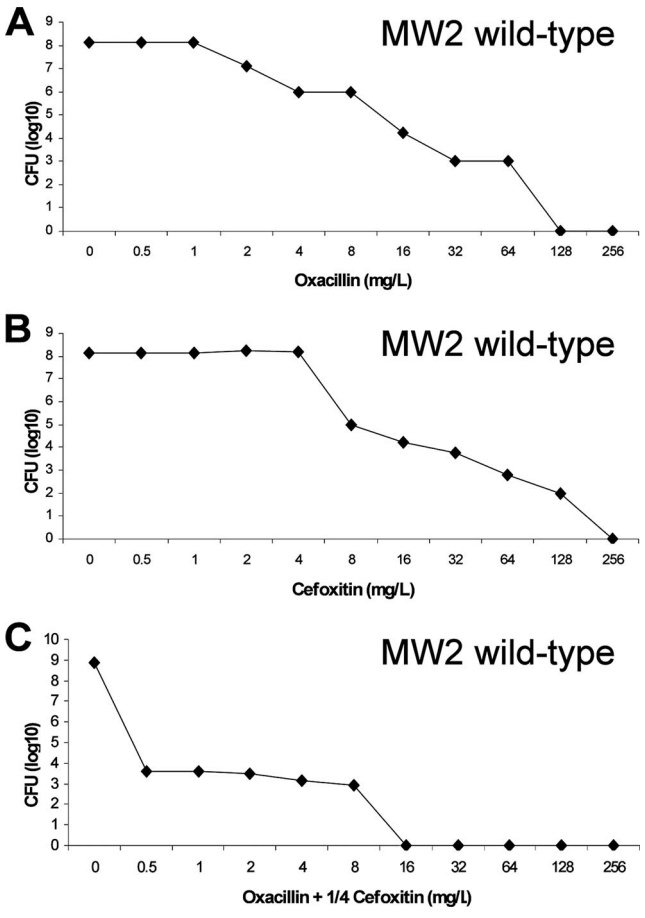


FIG. 6. Population analysis of susceptibility to oxacillin (A), cefoxitin (B), and oxacillin combined to one-fourth the MIC of cefoxitin (C) for wild-type strain MW2. Antibiotic susceptibilities of strains were determined in cultures grown overnight plated onto agar containing different concentrations of oxacillin, cefoxitin, or one-fourth the MIC of cefoxitin with twofold dilutions of oxacillin and incubated at 37°C for 48 h.

than the parental MW2 strain (Δ*pbp4* with MIC of 4 μg/ml versus 64 μg/ml in MW2), where *mecA* is under the regulation of the *blaIRZ* operon on plasmid pMW2. This observation suggests that the constitutive expression of PBP2A did not contribute to oxacillin resistance in community-acquired strains in the absence of PBP4.

Although the loss of *pbp4* in CA-MRSA strain MW2 affects the transcription of *pbp2* in cells challenged with oxacillin, this was not the only factor contributing to the resistance mechanism, since the complementation of *pbp2* in *trans* did not restore β-lactam resistance to the parental level in either the *pbp4* or the *pbp3 pbp4* mutant. We have ruled out the possibility that the difference in *pbp2* transcription between COL and MW2 might be due to polar effects upstream and/or downstream of the deleted Δ*pbp4* region. We also evaluated the only PBP2 regulator described so far, i.e., the two-component regulatory system *vraSR* (36), which was found to be upregulated in all strains in the presence of oxacillin. We are currently investigating if genes present in either the type I SCC*mec* of COL or type IV SCC*mec* could be the reason behind such a striking difference not only in *pbp2* expression but also in oxacillin resistance between CA-MRSA and HA-MRSA isolates in the absence of PBP4.

Our data here confirm the previously reported cooperative activity of PBP2, PBP4, and PBP2A and add a link in transcription between *pbp4* and the innate *pbp2* (37). Our results also showed that the increase in sensitivity to β-lactams associated with the loss of *pbp4* is not due to increased autolysis. HPLC analysis of peptidoglycan muropeptides confirmed that the loss of PBP4 in MW2 was associated with a reduction in peptidoglycan cross-linking and, hence, an increase in the percentage of monomeric, dimeric, and trimeric muropeptides compared to that of the parental strain (Fig. 5). We also carried out muropeptide analysis of *pbp4* mutants of HA-MRSA strain COL and methicillin-sensitive *S. aureus* strain Newman and found a lesser reduction in the percentage of highly cross-linked muropeptides than with the *pbp4* mutant of MW2 (data not shown).

Given the role of PBP4 in mediating resistance to β-lactams in CA-MRSA strains, we assessed whether a strategy against PBP4 could lead to a plausible therapeutic approach against them. Cefoxitin, which binds PBP4 irreversibly, was found to be synergistic with oxacillin at killing CA-MRSA strains compared to oxacillin alone. In the presence of one-fourth the MIC of cefoxitin, the MIC of oxacillin went down 64-fold, from 64 μg/ml to 1 μg/ml; an even more significant drop was found for MBC with both USA300 and MW2, from 256 μg/ml to <1 μg/ml. The fact that both cefoxitin and oxacillin have been widely used for years, at MIC and MBC concentrations that are easily achievable in vivo for CA-MRSA, strongly reinforces their therapeutic potential. The efficacy of this synergistic combination on other CA-MRSA isolates was subsequently tested on 200 MRSA isolates from Dartmouth-Hitchcock Medical Center, Lebanon, NH, blinded with respect to the origin. All the isolates that were found to be community acquired with our genetic screen were sensitive to a combination of oxacillin and cefoxitin. The code was subsequently broken, confirming that all 30 isolates, designated by us as being CA-MRSA isolates, were obtained from patients with primary skin infections and annotated by the hospital as being CA-MRSA isolates. We further confirmed this approach by testing this combination on 30 additional MRSA isolates that caused CSSTI (Table 3) and that were positive for our genetic markers. These CSSTI isolates were again sensitive to the combined action of cefoxitin and oxacillin. There were only two isolates whose MICs for this combination were higher than those for the rest of the tested strains (MIC of oxacillin of 4 μg/ml compared to 1 μg/ml but still significantly below the maximum achievable concentration in vivo). The genetic reason for this small increase in these two strains is not entirely clear but may be due to a higher level of resistance to cefoxitin.

Collectively, our results clearly demonstrate that PBP4 is a key element in β-lactam resistance in CA-MRSA strains. We also showed that PBP2A, the product of *mecA*, is not the sole determinant for oxacillin resistance in these isolates. Accordingly, cefoxitin may be used in combination with synthetic penicillins to treat CA-MRSA infections. Additionally, this antibiotic profile on sensitivity to a combination of cefoxitin and oxacillin can also be used as a tool to screen clinical specimens for CA-MRSA strains. An understanding of the genetic determinants that account for the difference in resistance between CA-MRSA and HA-MRSA strains will be vital for the development of novel strategies against staphylococcal infections.

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