Properties of a Novel PBP2A Protein Homolog from *Staphylococcus aureus* Strain LGA251 and Its Contribution to the β-Lactam-resistant Phenotype

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Methicillin-resistant *Staphylococcus aureus* (MRSA) strains show strain-to-strain variation in resistance level, in genetic background, and also in the structure of the chromosomal cassette (SCCmec) that carries the resistance gene mecA. In contrast, strain-to-strain variation in the sequence of the mecA determinant was found to be much more limited among MRSA isolates examined so far. The first exception to this came with the recent identification of MRSA strain LGA251, which carries a new homolog of this gene together with regulatory elements mecI/mecR that also have novel, highly divergent structures. After cloning and purification in *Escherichia coli*, PBP2ALGA, the protein product of the new mecA homolog, showed aberrant mobility in SDS-PAGE, structural instability and loss of activity at 37 °C, and a higher relative affinity for oxacillin as compared with cefoxitin. The mecA homolog free of its regulatory elements was cloned into a plasmid and introduced into the background of the β-lactam-susceptible *S. aureus* strain COL-S. In this background, the mecA homolog expressed a high-level resistance to cefoxitin (MIC = 400 μg/ml) and a somewhat lower resistance to oxacillin (minimal inhibitory concentration = 200 μg/ml). Similar to PBP2A, the protein homolog PBP2ALGA was able to replace the essential function of the *S. aureus* PBP2 for growth. In contrast to PBP2A, PBP2ALGA did not depend on the transglycosylase activity of the native PBP2 for expression of high level resistance to oxacillin, suggesting that the PBP2A homolog may preferentially cooperate with a monofunctional transglycosylase as the alternative source of transglycosylase activity.

Methicillin-resistant *Staphylococcus aureus* (MRSA) was first reported in England in 1961, soon after introduction of the penicillinase-resistant β-lactam antibiotic methicillin into clinical practice. Methicillin resistance is the single most important clinical resistance trait acquired by *S. aureus*. It is able to confer cross-resistance to virtually all β-lactam antibiotics, which represent the single most commonly prescribed class of antibacterial agents. Since their first appearance in 1961, epidemic strains of MRSA have spread worldwide in hospitals and in the community, and MRSA infections continue to present one of the major challenges to the control of infectious diseases in our era.

Methicillin resistance in *S. aureus* is mediated by an acquired penicillin-binding protein (PBP), named PBP2A (2), which has an extremely low reactivity with β-lactam antibiotics because of two factors: a high association constant for the antibiotic in the non-covalent complex and a poor first-order rate constant for the acylation of the protein by the antibiotic (3).

PBP2A is a peptidoglycan transpeptidase that, in cooperation with the transglycosylase domain of PBP2 of *S. aureus*, can catalyze cell wall biosynthesis in the presence of β-lactam antibiotics, thus enabling survival and growth of the bacteria (2, 4). PBP2A is encoded by the imported *mecA* gene, which is incorporated into the *S. aureus* chromosome as part of a large heterogeneous mobile genetic element, the staphylococcal cassette chromosome mec, or SCCmec (5). The SCCmec structure shows extensive variation from one MRSA clone to another. In contrast, strain-to-strain variation in the sequence of the *mecA* determinant is much more limited.

The abbreviations used are: MRSA, methicillin-resistant *Staphylococcus aureus*; PBP, penicillin-binding protein; B-PER, bacterial protein extraction reagent; IPTG, isopropyl β-D-thiogalactopyranoside; MIC, minimal inhibitory concentration; TGase, transglycosylase.

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* Background: The recently isolated MRSA LGA251 has low resistance and carries a new mecA homolog.

* Results: PBP2ALGA, the protein product of the new mecA, showed a “preference” for penicillins and instability at 37 °C. mecALGA251 introduced into susceptible *S. aureus* allowed expression of high-level resistance.

* Significance: This study provides insights into the relationship between structure and function of PBP2A-like proteins.

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†1 The abbreviations used are: MRSA, methicillin-resistant *Staphylococcus aureus*; PBP, penicillin-binding protein; B-PER, bacterial protein extraction reagent; IPTG, isopropyl β-D-thiogalactopyranoside; MIC, minimal inhibitory concentration; TGase, transglycosylase.

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The first exception to this was provided by studies on the recently described bovine *S. aureus* isolate LGA251 that showed low-level resistance to the β-lactam antibiotics cefoxitin and oxacillin, suggesting the presence of a MRSA strain (6). However, attempts to amplify the *mecA* determinant using PCR established for detection of the *mecA* gene carried by all well characterized MRSA strains were unsuccessful, and full genome sequencing of LGA251 identified a novel *mecA* homolog, *mecA*<sub>LGA251</sub>, in the strain. Strain LGA251 also carried a *mecl/mecR1* operon, a *blaZ*, and a SCC*mec* named SCC*mec* XI, each representing novel structures that have not been seen before in other MRSA isolates. Subsequently, *S. aureus* isolates carrying the same new SCC*mec* homolog have also been detected in some human isolates (6–8).

The purpose of the studies described here was twofold 1) to elucidate the properties of the novel PBP2A protein homolog produced by *S. aureus* LGA251 and compare them to PBP2A expressed in the MRSA strain COL and 2) to compare the mechanism of β-lactam resistance exhibited by strain LGA251 to that of the resistance mechanisms identified in MRSA strains that carry the typical *mecA* determinant.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Plasmids**—The characteristics of bacterial strains and plasmids used in this study are described in supplemental Table S1. *S. aureus* strains were grown in tryptic soy broth (Difco Laboratories) or in tryptic soy agar (Difco Laboratories) at 30 °C or 37 °C with aeration, depending on the strain and on the experiment. *Escherichia coli* strains were grown in Lumir Bertani broth (Difco Laboratories) or in Lumir-Bertani agar (Difco Laboratories) with aeration at 37 °C. Recombinant *E. coli* strains were selected and maintained with ampicillin at 100 µg/ml or with kanamycin at 30 µg/ml. Recombinant *S. aureus* strains were selected and maintained in a medium supplemented with the appropriate antibiotics (erythromycin at 10 µg/ml, chloramphenicol at 10 µg/ml, neomycin at 50 µg/ml, or kanamycin at 50 µg/ml). Isopropyl β-D-thiogalactopyranoside (IPTG, 0.1 mM) or CdCl<sub>2</sub> (0.2 mM) were added to the medium to induce the transcription of *pbpB* or *mecA*<sub>LGA251</sub>, respectively, in recombinant *S. aureus* strains.

**Antibiotic Susceptibility Tests**—The susceptibility of *S. aureus* strains to β-lactam antibiotics was determined by Etest (bioMérieux, Inc.) and/or by population analysis profiles. The Etest was performed by spreading a small aliquot of overnight cultures diluted to an A<sub>620</sub> of 0.08 on Mueller Hinton II agar plates (BD Biosciences), followed by placing Etest strips on the plates. Minimal inhibitory concentration (MIC) values of β-lactam drugs were evaluated after 24-h incubation at 30 °C and/or 37 °C. The population analysis was carried out on tryptic soy agar plates containing increasing concentrations of β-lactam antibiotics (and supplemented, if appropriate, with 0.1 mM of IPTG or 0.2 mM of CdCl<sub>2</sub>) as described previously (9–10). Colony-forming units were counted after 48-h incubation of the plates at 30 °C or 37 °C.

**Cloning of mecA Genes in *E. coli***—The *mecA* and *mecA*<sub>LGA251</sub> genes encoding PBP2A and the PBP2A homolog (PBP2A<sub>LGA</sub>), respectively, were cloned into an expression vector, pET24d(+)

(EMD Chemicals, Inc.) to overexpress and purify soluble PBP2A proteins in *E. coli*. Each gene was amplified by PCR with primers (CHismecAF and CHismecAR for *mecA*, CHismec ALGAF and CHismecALGAR for *mecA*<sub>LGA251</sub>) from *S. aureus* COL and *S. aureus* LGA251, respectively. All primers used in this study are listed in supplemental Table S2. The primers were designed to remove an N-terminal transmembrane region and add a His tag at the C termini of encoded proteins. PCR products were ligated into the Ncol and Xhol sites of pET24d(+). The recombinant plasmids were introduced into *E. coli* Tuner(DE3) and *E. coli* Rosetta(DE3), supplementing six rare codons (EMD Chemicals, Inc.) to produce soluble forms of C-terminal His-tagged PBP2A proteins. All sequences of the recombinant genes were confirmed by sequencing at Genewiz, Inc.

**Purification of C-terminal His-tagged PBP2A Proteins**—Overnight cultures carrying the recombinant plasmids were inoculated in 250 ml of Luria Bertani broth medium containing 50 µg/ml of kanamycin. Cells were grown at 37 °C until the A<sub>600</sub> had reached 0.6, at which time the cultures were cooled to room temperature. The expression of proteins was induced by adding IPTG at a final concentration of 0.4 mM, followed by incubation at 25 °C for 18 h with vigorous shaking at 200 rpm.

PBP2A was purified by a single step of Ni-affinity chromatography with a B-PER 6× His fusion protein purification kit (Thermo Fisher Scientific, Inc.), following the procedure of the manufacturer. In contrast, PBP2A<sub>LGA</sub> had to be isolated and refolded from inclusion bodies (11). Inclusion bodies were solubilized in 100 mM Tris-HCl (pH 8.0), with 50 mM glycerine and 6 mM guanidine chloride. Denatured proteins were refolded by stepwise dialysis in 100 mM Tris-HCl (pH 8.0), 400 mM L-arginine and 0.2 mM PMSF, supplemented with 4.0 M, 2.0 M, 1.0 M, 0.5 M and 0.0 M urea. Each dialysis step was performed for 24 h. At the final step of dialysis, 10% glycerol was added, and the refolded protein was centrifuged at 10,000 × g for 15 min to remove the precipitant. The supernatant was applied to a nickel-chelated agarose column for further purification. The full length of purified proteins was confirmed by peptide mass spectrometry with partial trypsin digestion at the Proteomics Resource Center (The Rockefeller University) and by immunoblotting with an anti-6× His antibody. In a parallel sample, PBP2A was deliberately denatured and refolded following the same procedure as for PBP2A<sub>LGA</sub> to make it fully comparable with the preparation of PBP2A<sub>LGA</sub>.

**Introduction of the mecA<sub>LGA251</sub> Gene into the *S. aureus* Strain COL-S and into a pbpB Conditional Mutant of COL-S**—To clone the *mecA*<sub>LGA251</sub> gene into a shuttle vector, pBCB8 equipped with a cadmium-inducible promoter (12), the gene was amplified by PCR using the primers mecALGAF5 and mecALGAR6 from LGA251 (supplemental Table S2). The product was ligated into the SacI and EcoRI sites of the pBCB8 plasmid. The recombinant plasmid was then introduced into *E. coli* DC10B (13) and was named pBCB8::mecA<sub>LGA251</sub>. The nucleotide sequence was confirmed by Macrogen Sequencing Services. Next, the recombinant plasmid pBCB8::mecA<sub>LGA251</sub>

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R. Sobral and M. Pinho, unpublished data.
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was introduced into the restriction-deficient *S. aureus* strain RN4220 (14) by electroporation (15), followed by transduction (16) using phage 80α into the oxacillin-susceptible *S. aureus* strain COL-S or into a pbpB conditional mutant of COL-S (17) to produce strains COL-SLGA$_{\text{meca}}$ and COL-S$_{\text{spec}}$-pbpB$_{\text{LGA-meca}}$, respectively, as described previously. In the transductants, transcription of the meca$_{\text{LGA251}}$ gene was under the control of the cadmium-inducible promoter.

**Preparation of Staphylococcal Membrane Proteins**—To determine the activity of PBP2A proteins associated with the bacterial plasma membrane, *S. aureus* strains COL and COL-S were grown at 37 °C in 250 ml of tryptic soy broth. *S. aureus* strains LGA251 and COL-S$_{\text{LGA-meca}}$ were grown at 30 °C. All strains were harvested when A$_{600}$ had reached 0.5, washed, and resuspended in 5 ml of 20 mm Tris-Cl (pH 7.6) containing 1× Halst protease inhibitor mixture (Thermo Fisher Scientific, Inc.) containing 50 μg/ml lysisostaphin, 50 μg/ml lysozyme, 40 μg/ml DNase I, and 40 μg/ml RNase A. Cells were incubated at room temperature for 30 min and disrupted by sonicating. The suspensions were centrifuged at 8000 × g for 15 min to remove unbroken cells, and the supernatants were transferred to fresh ultracentrifuge tubes. Membrane fractions were collected by centrifugation at 100,000 × g for 1 h. The collected membranes were resuspended in 20 mm Tris-Cl (pH 7.6) and stored at −70 °C (18, 19). The concentration of total membrane proteins was determined by BCA assay.

**Detection of PBP2A and PBP2A$_{\text{LGA}}$ by Western Blotting**—Western blotting with a rabbit anti-PBP2A antibody was used for detection of the two proteins both in the purified His-tagged form and also in membrane preparations. The procedure was as described previously (20–21) with a few modifications, which were as follows. The primary antibody was used after dilution of 1:10,000, and the secondary antibody was the HRP-coupled anti-rabbit antibody (10 μg/ml, Thermo Fisher Scientific, Inc.), which was diluted 1:150. The ChromPure human IgG Fc fragment was not added for detecting the purified proteins. The rabbit anti-6× His antibody (dilution, 1:2000; Rockland, Inc.) was used only for detection of His-tagged proteins. The chemiluminescent substrate for HRP was Supersignal West Pico chemiluminescent substrate (Thermo Fisher Scientific, Inc.).

**Determination of CD Spectra**—Purified PBP2A and PBP2A$_{\text{LGA}}$ were desalted with a Micro Bio-Spin 6 column (Bio-Rad) following the instructions of the manufacturer. The proteins were diluted to 1.5 μM in 10 mm sodium phosphate (pH 7.0) and incubated at 25 °C and 37 °C for 20 min prior to the CD measurement. The CD spectra were recorded on an AVIV-62 CD spectrometer (AVIV Biomedical, Inc.; 0.2-cm path length) to examine conformations of the two proteins under different experimental conditions (22–24).

**Determination of the Activity and Thermostability of PBP2A and PBP2A$_{\text{LGA}}$**—Purified proteins (25 μg/ml) were preincubated in 20 mm sodium phosphate (pH 7.0) at 25, 30, and 37 °C for various periods of time (0, 2, 5, 10, and 20 min) prior to addition of Bocillin FL (40 μg/ml, Invitrogen), followed by further incubation at the indicated temperatures for 15 min. Reactions were quenched by adding SDS loading buffer and boiling for 3 min. Samples were applied to 10% gels for SDS-PAGE to visualize Bocillin FL-labeled PBP2A proteins (18).

**RESULTS**

**Properties of Strain LGA251**—To confirm the MIC values reported previously (16 μg/ml for oxacillin and 32 μg/ml for cefoxitin) (6), the resistance of strain LGA251 to oxacillin and cefoxitin was determined by Etest at 30 °C and 37 °C. The MIC of LGA251 was higher for cefoxitin (24 μg/ml) as compared with oxacillin (6 μg/ml) at 37 °C. The corresponding MIC values determined at 30 °C were 64 μg/ml for cefoxitin and 16 μg/ml for oxacillin (supplemental Fig. S1). The presence of numerous isolated colonies in the “clear space” of the Etest indicates that strain LGA251 is heteroresistant (28).

Initial attempts to characterize the novel β-lactam resistance mechanism in strain LGA251 were unsuccessful. First, a Bocillin FL-binding assay was used to determine the presence of a penicillin-binding protein with low affinity for β-lactam antibiotics in strain LGA251. The assay was performed using membrane preparations of LGA251 grown in the absence and presence of cefoxitin (1.5 μg/ml) to induce transcription of meca$_{\text{LGA251}}$ gene. Only a very faint band corresponding to a PBP2A homolog was detected in membrane preparations prepared from cefoxitin-treated LGA251 (data not shown).

In another attempt, immunoblotting with a monoclonal antibody prepared against the N-terminal sequence of PBP2A from the MRSA strain COL (21) was used with strain LGA251. This assay produced a weak band using membrane preparations from cefoxitin-treated LGA251 within the molecular weight...
range where one would expect to find a PBP2A homolog (supplementary Fig. S2). Testing for a transcript of the mecA_LGA251 by Northern blotting also produced a very weak band only (data not shown).

These observations indicated that determining properties of the new mecA homolog and relating them to the mechanism of resistance in strain LGA251 would be difficult, presumably because of the powerful repression of transcription of mecA_LGA251 by the novel mecI/mecR1 system present in strain LGA251.

To bypass this problem, we chose two different experimental approaches. The first approach involved cloning and purifying the protein product of mecA_LGA251 (to be named throughout this paper as PBP2A_LGA) in E. coli along with PBP2A and comparing their properties by in vitro assays.

The second approach involved cloning the mecA_LGA251 free of its regulatory elements (mecI/mecR1) into a plasmid followed by introducing it into the background of the susceptible S. aureus strain COL-S and determining various aspects of the antibiotic resistance mechanisms in the background of this well characterized S. aureus strain (29).

Characterization of PBP2A and PBP2A_LGA after Cloning and Purification from E. coli—Purification and comparison of properties of C-terminal His-tagged PBP2A and PBP2A_LGA in E. coli. After overexpressing in E. coli, PBP2A isolated from the MRSA strain COL remained soluble. In contrast, PBP2A_LGA cloned from LGA251 formed a precipitate in inclusion bodies. After solubilization and refolding of PBP2A_LGA, both proteins were purified through Ni-affinity chromatography for their C-terminal His-tags and were compared for a variety of properties in vitro.

Tests with SDS-PAGE indicated that PBP2A and PBP2A_LGA had higher than 95% purity after elution from the nickel column (Fig. 1A).

Molecular Size and Electrophoretic Mobility of PBP2A_LGA—PBP2A_LGA ran faster than PBP2A on the gel under the experimental conditions used even though the two proteins had the same molecular weight of 75 kDa deduced from their amino acid sequences.

To exclude the possibility that PBP2A_LGA may be a truncated peptide, the full length of PBP2A_LGA was confirmed by immunoblotting with both anti-PBP2A antibody and anti-6× His antibody. The former was produced for an epitope of PBP2A at the beginning of its N-terminal domain5, and the latter was for the His tag carried at the C termini of the purified proteins. Although the luminescence of PBP2A_LGA was weaker than that of PBP2A for both antibodies, the chemiluminescence by the two antibodies was detected at the same position, marked by Coomassie Blue staining (Fig. 1B). Therefore, the observed lower molecular size of PBP2A_LGA on SDS-PAGE was not due to truncation of the peptide nor due to immature translation. A mass spectrometric analyses of the His-tagged PBP2A proteins

5 Although prepared against PBP2A, the monoclonal antibody was also able to recognize PBP2A_LGA because PBP2A and PBP2A_LGA share nine of the 20 amino acids of the epitope, thus allowing a partial recognition by the antibody.
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**Figure 2.** Optimal temperatures for the activity and the stability of PBP2A and PBP2A<sub>LGA</sub>. A, purified proteins were incubated at different temperatures (25 °C, 30 °C and 37 °C) for various time periods, after which the activity of the preparations was determined by Bocillin FL binding assay. Lanes I and II represent the fluorescence of Bocillin FL-bound proteins and the amount of loaded proteins, respectively. B, the activity of PBP2A<sub>LGA</sub> was plotted as a function of preincubation time. The activity of each protein was normalized by dividing the fluorescent intensity of each lane by the corresponding protein amount. The value at 0 min was set as 100%. PBP2A<sub>LGA</sub> dramatically lost its activity at 37 °C. The experiment was independently performed in triplicate.

PBP2As recovered by the two different procedures were indistinguishable (data not shown).

Comparison of PBP2A and PBP2A<sub>LGA</sub> for Affinities to Oxacillin and Cefoxitin—The proteins were preincubated with increasing concentrations (from 0 to 2000 μg/ml) of oxacillin or cefoxitin for 15 min, after which the amount of protein that remained free (i.e. was not acylated by oxacillin or cefoxitin) was determined using the Bocillin FL binding assay. The antibiotic susceptibility profile of transductants was confirmed by the anti-PBP2A antibody, and the protein appeared at the same position, with higher mobility than PBP2A, on the polyacrylamide gel, similar to the purified protein (supplemental Fig. S2).

The antibiotic susceptibility profile of transductants was evaluated by population analysis using either oxacillin or cefoxitin as the antibacterial agents in the absence and the presence of 0.2 μM CdCl₂ as the inducer.

In the absence of CdCl₂, COL-S<sub>LGAmece</sub> exhibited a low and heterogeneous resistance with an MIC value of 12.5 μg/ml for both antibiotics. In contrast, with the CdCl₂ inducer added to the medium, COL-S<sub>LGAmece</sub> showed a high and homogeneous resistance to both antibiotics with a resistance level that was higher for cefoxitin (MIC = 400 μg/ml at 37 °C and 800 μg/ml at 30 °C) than for oxacillin (MIC = 200 μg/ml at 37 °C and 400 μg/ml at 30 °C) (Fig. 5, A and B).

The construct COL-S<sub>LGAmece</sub> also showed uniformly high MIC values in the range of several hundred μg/ml for a number of other β-lactam antibiotics, including cloxacillin, cephradine,

weights of oxacillin and cefoxitin are very similar, 441.43 and 449.43, respectively.)

The Far UV CD spectra for PBP2A and PBP2A<sub>LGA</sub>—The CD spectra of PBP2A and PBP2A<sub>LGA</sub> were measured to examine if their conformations were affected by temperature because the former showed optimal activity at 37 °C, and the latter was unstable at this temperature.

PBP2A showed a more relaxed structure at 37 °C than at 25 °C, indicating that the α-helicity of the protein was reduced at the higher temperature (Fig. 4). In contrast, the CD spectrum of PBP2A<sub>LGA</sub> exhibited a very different pattern at 37 °C because of precipitation of the protein during 20 min of preincubation, indicating the structural instability. The CD spectrum of PBP2A at 37 °C was similar to that of PBP2A<sub>LGA</sub> at 25 °C, temperatures at which these two proteins exhibit their maximum activity. This finding was consistent with the results obtained with the Bocillin FL binding assay.

Cloning of mec<sub>LGA251</sub> Free of Its Regulatory Elements and Expression in the Background of S. aureus strain COL-S—In a second approach to characterize the mec<sub>LGA251</sub> of S. aureus strain LGA251, the gene was cloned in a staphylococcal plasmid that was then used to introduce the gene into the background of an antibiotic-susceptible S. aureus strain that has no penicillinase and is also free of the regulatory elements mecl/mecR1. As such a recipient we chose strain COL-S, which was generated in the laboratory from the highly oxacillin-resistant clinical MRSA isolate COL through removing the entire SCCmec element by precise excision (29). It was shown earlier that introduction of plasmid-borne copies of the mecA gene into strain COL-S could produce bacteria with a high level and homogeneous resistance to oxacillin (17, 21).

The mec<sub>LGA251</sub> was cloned into plasmid pBCB8 equipped with a cadmium-inducible promoter (12, 30), and strain COL-S was transduced with pBCB8::mecA<sub>LGA251</sub>. This construct, COL-S<sub>LGAmece</sub>, was used in a series of experiments to explore the functioning of the mecA homolog free of its regulatory elements.

The production of PBP2A<sub>LGA</sub> in transductants was confirmed by the anti-PBP2A antibody, and the protein appeared at the same position, with higher mobility than PBP2A, on the polyacrylamide gel, similar to the purified protein (supplemental Fig. S2).

The antibiotic susceptibility profile of transductants was evaluated by population analysis using either oxacillin or cefoxitin as the antibacterial agents in the absence and the presence of 0.2 μM CdCl₂ as the inducer.

In the absence of CdCl₂, COL-S<sub>LGAmece</sub> exhibited a low and heterogeneous resistance with an MIC value of 12.5 μg/ml for both antibiotics. In contrast, with the CdCl₂ inducer added to the medium, COL-S<sub>LGAmece</sub> showed a high and homogeneous resistance to both antibiotics with a resistance level that was higher for cefoxitin (MIC = 400 μg/ml at 37 °C and 800 μg/ml at 30 °C) than for oxacillin (MIC = 200 μg/ml at 37 °C and 400 μg/ml at 30 °C) (Fig. 5, A and B).

The construct COL-S<sub>LGAmece</sub> also showed uniformly high MIC values in the range of several hundred μg/ml for a number of other β-lactam antibiotics, including cloxacillin, cephradine,
and cefoxitin, representing antibiotics with unique binding affinities for various *S. aureus* PBPs (data not shown).

**Bocillin FL Binding Assay with Membrane Preparations of COL and COL-SLGA mecA**—Membrane preparations from COL and COL-SLGA mecA were exposed to 1.0 mg/ml of clavulanate to saturate all PBPs except the PBP2A proteins (25–27), and the binding affinities of membrane-anchored PBP2A and PBP2A SLGA to antibiotics were examined using various concentrations of oxacillin and cefoxitin (Fig. 6, A and B). Gels were scanned for fluorescence using the Bocillin FL binding assay (Fig. 6, A and B, upper panels) and developed by Coomassie Blue staining (lower panels). The assay in the absence of clavulanate (see lanes marked NC) allowed visualization of the native *S. aureus* PBP2s 1 through 3, whereas PBP2A was adjusted to a more active form. The measurement was carried out three times independently.

![Comparison of a Novel PBP2A SLGA with PBP2A](image)

**FIGURE 3.** Measurement of the affinities of PBP2A and PBP2A SLGA for two structurally different β-lactam antibiotics. The two proteins were preincubated with different concentrations of oxacillin and/or cefoxitin for 15 min, after which the fraction of the proteins that remained non-acylated was determined by Bocillin FL binding assay. A, the decreasing fluorescence by β-lactam antibiotics on SDS-PAGE. B, the plot for the percentage of unbound proteins as a function of oxacillin concentration. C, the plot for the percentage of unbound proteins as a function of cefoxitin concentration. IC₅₀ values of oxacillin and cefoxitin were calculated from the plots in B and C. The affinity was independently evaluated in triplicate.

**FIGURE 4.** The far UV CD spectra for PBP2A and PBP2A SLGA at 25 °C and 37 °C. The proteins were preincubated for 20 min at the indicated temperatures prior to CD measurement. The conformation of PBP2A SLGA was disrupted at 37 °C, whereas PBP2A was adjusted to a more active form. The measurement was carried out three times independently.

**FIGURE 5.** High-level antibiotic resistance produced in the β-lactam-susceptible *S. aureus* strain COL-S by introducing plasmid-borne copies of the mecALGA251. The mecA determinant of strain LGA251 was cloned free of its mec/mecR regulatory elements into the cadmium-inducible plasmid pBCB8 and transduced into strain COL-S. The antibiotic resistance of the transductants was determined by population analysis. Colony-forming units (CFU) were calculated by counting colonies after 48 h of incubation on tryptic soy agar plates supplemented with 0.2 μM CdCl₂ at 37 °C and 30 °C. The closed symbols indicate the resistance of strain COL-SLGA mecA to oxacillin (■) and cefoxitin (●) at 37 °C. The open symbols indicate the resistance of strain COL-SLGA mecA to oxacillin (○) and cefoxitin (●) at 30 °C. The triangles represent the susceptibility profile of strain COL-S to oxacillin (▲) and cefoxitin (●).
survival and growth of β-lactam-susceptible S. aureus (31). It has also been shown that a conditional mutant of PBP2 was able to grow for considerable periods of time in the absence of the inducer, provided that the strain was a MRSA, i.e. carried the PBP2A protein (31). This finding demonstrated that the antibiotic resistance protein PBP2A can also perform, at least partially, the normal biosynthetic functions of PBP2.

This experiment was repeated using the strain expressing PBP2ALGA. Fig. 7 shows that PBP2ALGA was able to perform this surrogate function of PBP2A. All bacterial strains used in this experiment had the common background of COL-S spac::pbpB, which carries a conditional mutation in PBP2 inducible by IPTG (31). Growth of the control strain COL-S spac::pbpB showed absolute dependence on the IPTG inducer, whereas growth of COL-S spac::pbpB carrying plasmid-borne copies of mecA LGA251 (COL-S spac::pbpB/LGA mecA) was able to grow in the absence of IPTG, provided that the medium was supplemented with 0.2 μM cadmium chloride.

PBP2ALGA Does Not Require a Functional PBP2 for Optimal Expression of Resistance to Oxacillin—It was demonstrated earlier that expression of high-level oxacillin resistance required not only PBP2A but a fully functional PBP2 as well because restricting the amounts of PBP2 in a conditional mutant by suboptimal concentrations of the IPTG inducer produced only heteroresistant phenotypes in which the MIC of the majority cells was proportional to the concentration of the IPTG inducer in the growth medium (31–32). The results illustrated in Fig. 8 show a striking difference when the same experiment was repeated in the pbpB conditional mutant of COL-S carrying the plasmid-borne copy of mecA_LGA251 strain COL-S spac::pbpB/LGA mecA was able to express a high level of oxacillin resistance, even in the complete absence of IPTG from the growth medium.

**DISCUSSION**

The method most frequently used for identification of MRSA isolates has been testing for the presence of a mecA determinant
Comparison of a Novel PBP2A<sub>LGA</sub> with PBP2A

Has a molecular weight of 81 kDa as deduced from its amino acid sequence, yet in SDS-PAGE this protein cannot be separated from PBP2A, which has the molecular size of 76 kDa. On the basis of the crystal structure of PBP2, it has been reported that the protein contains a membrane-embedded region at its transglycosylase domain (33), indicative of high hydrophobicity, which may explain its aberrant molecular size in SDS-PAGE.

In the case of PBP4 of <i>S. sciuri</i>, a putative evolutionary relative of PBP2A, the protein was shown to run slower than PBP2A in SDS gels, even though its polypeptide (666 amino acids) is only two amino acids shorter than PBP2A (668 amino acids) (17, 21). The chemiluminescence of PBP2A<sub>LGA</sub> is substantially weaker than that of PBP2A, indicating incomplete denaturation of PBP2A<sub>LGA</sub> by SDS under the conditions of standard SDS-PAGE. These observations suggest that the aberrant mobility of PBP2A<sub>LGA</sub> may be related to either its higher hydrophobicity or to the lack of full denaturation under the conditions of SDS-PAGE.

The second difference between PBP2A and PBP2A<sub>LGA</sub> is the different temperature optimum of their activity and the thermosensitivity of the PBP2A<sub>LGA</sub> protein. The CD spectra of the two proteins were shown to be similar at their respective temperature optima, but the structure of PBP2A<sub>LGA</sub> was shown to collapse at the higher temperature, indicating that conformational changes that occur in this protein are responsible for its instability and loss of activity.

The third difference between the two proteins is their different affinity for two β-lactam antibiotics. In contrast to PBP2A, which has somewhat higher affinity (1.4-fold) for cefoxitin than oxacillin, PBP2A<sub>LGA</sub> has a higher affinity (2.5-fold) for oxacillin as compared with cefoxitin. This opposite binding preference of the two proteins is the result of the large difference in binding affinity of these proteins for penicillins such as Bocillin FL or oxacillin. PBP2A<sub>LGA</sub> binds to oxacillin 4-fold better than PBP2A. On the other hand, the binding affinity of the two proteins for a cephalosporin, cefoxitin, is almost identical. These findings suggest that the selective pressure on a hypothetical microorganism carrying the ancestral <i>mecA</i><sub>LGA251</sub> gene was most likely exposure to cephalosporin rather than to penicillins, which is consistent with the routine use of cephalosporins for treatment and prophylaxis of bovine mastitis in veterinary medicine<sup>6</sup>.

Expression of <i>mecA</i><sub>LGA251</sub> in <i>S. aureus</i> Strain COL-S—Introduction of plasmid-born copies of the <i>mecA</i><sub>LGA251</sub> into the <i>S. aureus</i> strain COL-S allowed testing the properties of PBP2A<sub>LGA</sub> in direct microbiological assays. The most surprising observation was that <i>mecA</i><sub>LGA251</sub> was able to produce a very high-level resistance to oxacillin and cefoxitin, far above the MIC values of the original strain, LGA251. We believe that this finding may reflect the absence of <i>mecI/mecR1</i> elements in this experimental system. In addition, as shown in Fig. 5, the higher MIC at the lower temperature and the higher MIC for cefoxitin over that of oxacillin observed in the original description of <i>mecA</i><sub>LGA251</sub> (6) was retained in this genetic background.

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<sup>6</sup>M. Holmes, personal communication.
Comparison of a Novel PBP2A<sub>LGA</sub> with PBP2A

Similar to PBP2A, PBP2A<sub>LGA</sub> was also able to replace the normal and essential transpeptidase function of the <i>S. aureus</i> PBP2 and support growth of a bacterial mutant in which transcription of the essential gene <i>pbpB</i> is suppressed (Fig. 7). Under these conditions, the second important biosynthetic function of PBP2, its transglycosylase (TGase) activity, is assumed to be provided by monofunctional glycosyltransf erases (Mgts) that are known to be induced when the production of PBP2 is inhibited (34).

Expression of High-level β-Lactam Resistance in <i>S. aureus Strain COL-S Carrying mecA<sub>LGA251</sub></i> and the TGase Function of PBP2—The two proteins PBP2A and PBP2A<sub>LGA</sub> showed clearly different behavior in another microbiological assay in which the capacity of these proteins was compared for generating high-level resistance to oxacillin in the common genetic background of strain COL-S. Early studies established that an optimal (high) level of oxacillin resistance required a collaborative function of PBP2A and the TGase domain of the resident PBP2 (4). In the experiment with strain COL-S carrying mecA<sub>LGA251</sub>, the optimal - high-level oxacillin resistance was obtained even under conditions when transcription of PBP2 was completely inhibited (Fig. 8). Because neither PBP2A nor PBP2A<sub>LGA</sub> has TGase activity, we interpret this finding as an indication of yet another difference between the two protein homologs. Although the transpeptidase of the PBP2A can function in collaboration with the TGase activity of PBP2, the protein homolog PBP2A<sub>LGA</sub> may “prefer” to collaborate with one of the monofunctional transglycosylases of <i>S. aureus</i> (34–36).

This preference for an alternative transglycosylase must ultimately reflect structural differences between PBP2A and PBP2A<sub>LGA</sub>, a full understanding of which will require comparison of their crystal structures.

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