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

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Background: The recently isolated MRSA LGA251 has low resistance and carries a new *mecA* homolog.
Results: $PBP2A_{LGA}$, the protein product of the new *mecA*, showed a "preference" for penicillins and instability at 37 °C. $mecA_{LGA251}$ 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.

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 (SCC*mec*) 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*, $PBP2A_{LGA}$, 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 $PBP2A_{LGA}$ was able to replace the essential function of the *S. aureus* PBP2 for growth. In contrast to PBP2A, $PBP2A_{LGA}$ 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)[3] was first reported in England in 1961 (1), 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 heterologous mobile genetic element, the staphylococcal cassette chromosome *mec*, or SCC*mec* (5). The SCC*mec* 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.

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[S] This article contains supplemental Figs. S1–S4, Tables S1 and S2, and references.
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[3] 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.

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*$_{LGA251}$, in the strain. Strain LGA251 also carried a *mecI/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 Luria Bertani broth (Difco Laboratories) or in Luria-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 $CdCl_2$ (0.2 μM) were added to the medium to induce the transcription of *pbpB* or *mecA*$_{LGA251}$, 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_{620} 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 μM of $CdCl_2$) 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 for Overexpression of His-tagged PBP2A and PBP2A$_{LGA}$—The *mecA* and *mecA*$_{LGA251}$ genes encoding PBP2A and the PBP2A homolog (PBP2A$_{LGA}$), 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*$_{LGA251}$) 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 NcoI and XhoI 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_{600} 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$_{LGA}$ 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 glycine and 6 M 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$_{LGA}$ to make it fully comparable with the preparation of PBP2A$_{LGA}$.

Introduction of the mecA$_{LGA251}$ Gene into the S. aureus Strain COL-S and into a pbpB Conditional Mutant of COL-S—To clone the *mecA*$_{LGA251}$ gene into a shuttle vector, pBCB8 equipped with a cadmium-inducible promoter[4] (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*$_{LGA251}$. The nucleotide sequence was confirmed by Macrogen Sequencing Services. Next, the recombinant plasmid pBCB8::*mecA*$_{LGA251}$

[4] R. Sobral and M. Pinho, unpublished data.

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$_{mecA}$ and COL-S$_{spac}$::pbpB$_{LGAmecA}$, respectively, as described previously. In the transductants, transcription of the *mecA*$_{LGA251}$ 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$_{LGAmecA}$ were grown at 30 °C. All strains were harvested when A_{620} had reached 0.5, washed, and resuspended in 5 ml of 20 mM Tris-HCl (pH 7.6) containing 1× Halt protease inhibitor mixture (Thermo Fisher Scientific, Inc.) containing 50 μg/ml lysostaphin, 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 sonication. 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-HCl (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$_{LGA}$ 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$_{LGA}$ 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$_{LGA}$—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). After electrophoresis, the gels were washed in water for 10 min and scanned on a Typhoon9400 image scanner (Molecular Dynamics, laser 488 nm, emission filter 526 nm). Fluorescent intensity was quantified by ImageQuant software.

Competition Assays for Determination of IC$_{50}$ values of PBP2A and PBP2A$_{LGA}$—Binding affinities of β-lactams for the two proteins were determined with a competition assay by incubating the proteins with various concentrations (from 0 to 2000 μg/ml) of β-lactams prior to adding Bocillin FL as a reporter. The assays for PBP2A and PBP2A$_{LGA}$ were performed at 37 °C and 25 °C, *i.e.* the temperatures at which the two proteins exhibited maximum activity and stability. Each protein was incubated with oxacillin and cefoxitin for 15 min, followed by addition of Bocillin FL. Samples were quenched and visualized as described above. IC$_{50}$ (μg/ml) values, defined as the concentration of antibiotics acylating 50% of each protein, were determined by the intensity of the bound Bocillin FL and were expressed as μg/ml because the sodium salts of oxacillin and cefoxitin, purchased from Sigma-Aldrich, have similar molecular weights (441.43 and 449.43, respectively). The IC$_{50}$ values were calculated from three independent assays.

To confirm the binding affinities of the two purified proteins to the antibiotics, PBP binding assays were carried out with each membrane preparation (100 μg) of COL and COL-SLGA$_{mecA}$ under the same conditions as used for the IC$_{50}$ determination. Membrane proteins were exposed to 1.0 mg/ml of clavulanate lithium (United States Pharmacopeia) for 10 min to saturate all PBPs except for PBP2A prior to adding oxacillin or cefoxitin (25–27). Proteins were separated on 8% gels of SDS-PAGE developed by Bocillin FL and were scanned for fluorescence.

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*$_{LGA251}$ 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 (supplemental 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. 1*A*).

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 domain[5], 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. 1*B*). 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

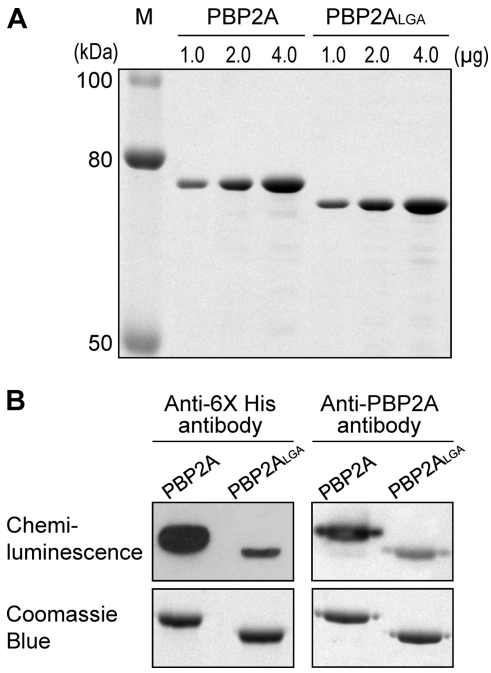

FIGURE 1. **SDS-PAGE and Western blotting of PBP2A and PBP2A$_{LGA}$.** *A*, the purity of the purified His-tagged proteins was checked by loading indicated amount on the 10% gel for SDS-PAGE and staining with Coomassie Blue R-250. *B*, the size of each protein was confirmed by immunoblotting with anti-6× His antibody and anti-PBP2A antibody. The *upper panels* and *lower panels* in *B* represent the intensity of the proteins developed by antibodies and Coomassie Blue, respectively. The chemiluminescence of PBP2A$_{LGA}$ was much weaker than that of PBP2A for both antibodies. *M* represents a size marker.

further confirmed that both proteins were full-length and had no posttranslational modification (supplemental Fig. S3).

Activity and Thermostability of PBP2A$_{LGA}$.—The two proteins were compared for activity and stability during incubation at three temperatures (25 °C, 30 °C and 37 °C) using the Bocillin FL-binding assay. Fig. 2*A* shows that the fluorescence of Bocillin FL-bound PBP2A exhibited maximum fluorescence at 37 °C and the activity was stable at this temperature. In contrast, PBP2A$_{LGA}$ appeared to have good fluorescence in a broad range of temperatures from 25 °C to 37 °C provided that the protein was exposed for only short periods of time. The activity of PBP2A$_{LGA}$ decreased dramatically with increasing incubation time at 37 °C. The reduction of activity at 37 °C seemed to be related to the instability of the protein at the higher temperature. Preincubation of the samples at 37 °C for 20 min prior to adding Bocillin-FL resulted in the loss of almost 80% of activity (Fig. 2*B*). PBP2A$_{LGA}$ showed stable and high activity at 25 °C. The initial fluorescence of PBP2A$_{LGA}$ was much stronger than that of PBP2A at their respective temperature optima. This observation suggests that PBP2A$_{LGA}$ has higher affinity to penicillins than PBP2A because Bocillin FL is a fluorescent derivative of penicillin V (18).

After cloning in *E. coli*, PBP2A$_{LGA}$ was recovered as a precipitate from inclusion bodies and required refolding before comparing it to PBP2A, which had remained soluble under these conditions. To exclude possible artifacts, PBP2A was deliberately denatured and refolded under the same condition as used for PBP2A$_{LGA}$, and the activity and stability of refolded PBP2A were compared with that of soluble PBP2A. The properties of

[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.

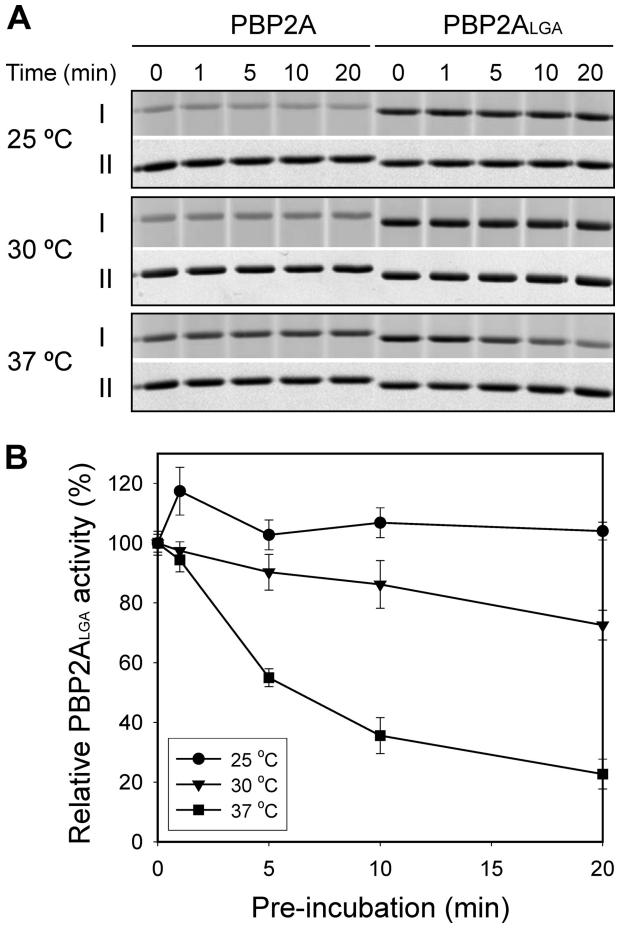

FIGURE 2. **Optimal temperatures for the activity and the stability of PBP2A and PBP2A$_{LGA}$.** *A*, purified proteins were incubated at different temperatures (25 °C, 30 °C and 37 °C) for various time periods, after which the activity of the preparations was determined by Bocillin FL binding assay. *Lanes I* and *II* represent the fluorescence of Bocillin FL-bound proteins and the amount of loaded proteins, respectively. *B*, the activity of PBP2A$_{LGA}$ 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$_{LGA}$ 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$_{LGA}$ for Affinities to Oxacillin and Cefoxitin—The proteins were preincubated with increasing concentrations (from 0 up 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 (Fig. 3), and the data were used for the calculation of the corresponding IC_{50} values. Although PBP2A$_{LGA}$ (IC_{50} = 85 ± 9 μg/ml) showed significantly higher binding affinity to oxacillin than PBP2A (IC_{50} = 340 ± 30 μg/ml), the two proteins exhibited virtually identical titration profiles for cefoxitin (IC_{50} = 214 ± 18 μg/ml for PBP2A$_{LGA}$ and 245 ± 27 μg/ml for PBP2A). PBP2A$_{LGA}$ showed a clearly higher affinity for oxacillin than for cefoxitin. PBP2A showed the opposite profile, exhibiting a marginally higher affinity to cefoxitin (supplemental Fig. S4). (IC_{50} values were expressed in μg/ml units because the molecular weights of oxacillin and cefoxitin are very similar, 441.43 and 449.43, respectively.)

The Far UV CD spectra for PBP2A and PBP2A$_{LGA}$—The CD spectra of PBP2A and PBP2A$_{LGA}$ 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$_{LGA}$ 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$_{LGA}$ 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 mecA$_{LGA251}$ Free of Its Regulatory Elements and Expression in the Background of S. aureus strain COL-S—In a second approach to characterize the *mecA*$_{LGA251}$ 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 *mecI/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 SCC*mec* 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 *mecA*$_{LGA251}$ was cloned into plasmid pBCB8 equipped with a cadmium-inducible promoter (12, 30), and strain COL-S was transduced with pBCB8::*mecA*$_{LGA251}$. This construct, COL-S$_{LGA}$*$_{mecA}$*, was used in a series of experiments to explore the functioning of the *mecA* homolog free of its regulatory elements.

The production of PBP2A$_{LGA}$ 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 $CdCl_2$ as the inducer.

In the absence of $CdCl_2$, COL-S$_{LGA}$*$_{mecA}$* exhibited a low and heterogeneous resistance with an MIC value of 12.5 μg/ml for both antibiotics. In contrast, with the $CdCl_2$ inducer added to the medium, COL-S$_{LGA}$*$_{mecA}$* showed a high and homogenous 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$_{LGA}$*$_{mecA}$* 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,

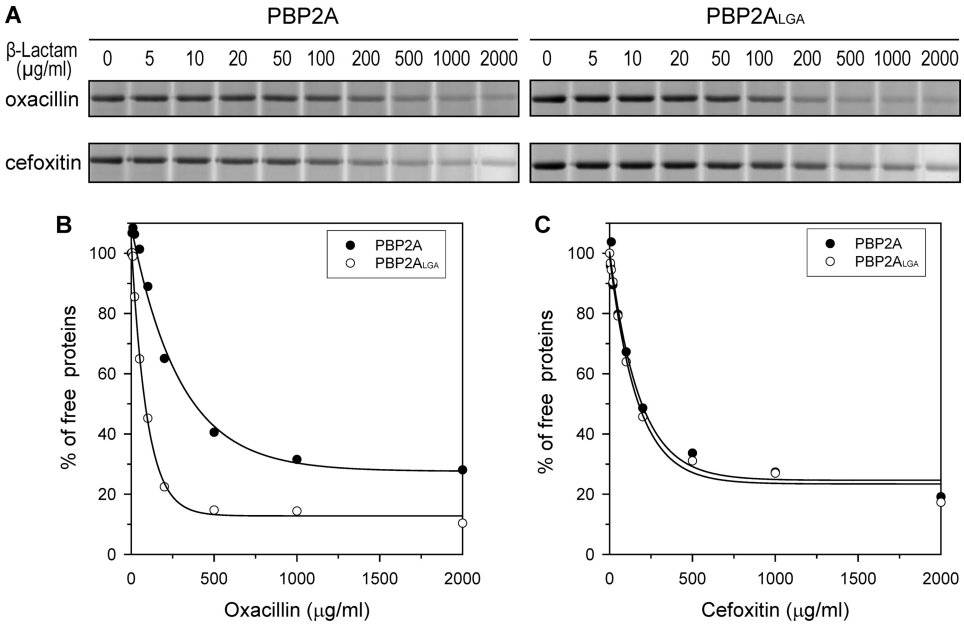

FIGURE 3. **Measurement of the affinities of PBP2A and PBP2A$_{LGA}$ for two structurally different β-lactam antibiotics.** The two proteins were preincubated with different concentrations of oxacillin and/or cefoxitin for 15 min, after which the fraction of the proteins that remained non-acylated was 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. IC$_{50}$ values of oxacillin and cefoxitin were calculated from the plots in *B* and *C*. The affinity was independently evaluated in triplicate.

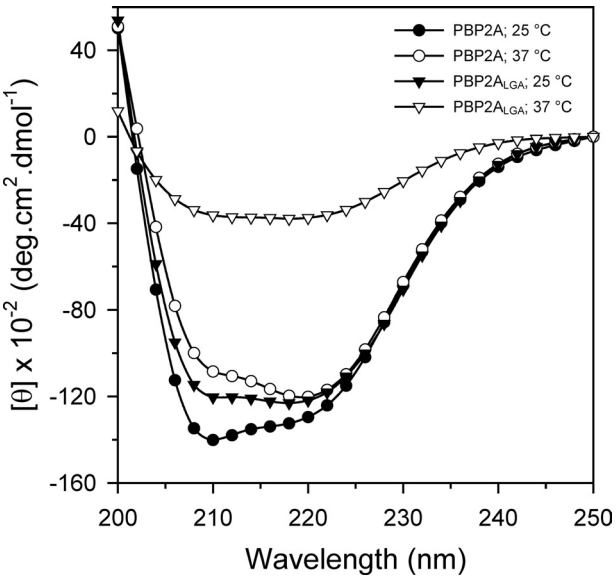

FIGURE 4. **The far UV CD spectra for PBP2A and PBP2A$_{LGA}$ at 25 °C and 37 °C.** The proteins were preincubated for 20 min at the indicated temperatures prior to CD measurement. The conformation of PBP2A$_{LGA}$ was disrupted at 37 °C, whereas PBP2A was adjusted to a more active form. The measurement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

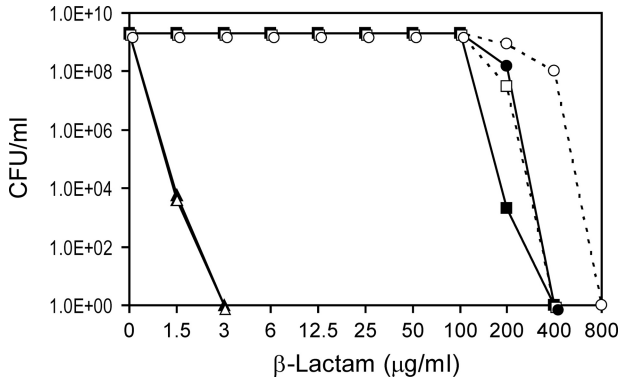

FIGURE 5. **High-level antibiotic resistance produced in the β-lactam-susceptible *S. aureus* strain COL-S by introducing plasmid-borne copies of the *mecA*LGA251.** The *mecA* determinant of strain LGA251 was cloned free of its *mecI/mecR1* regulatory elements into the cadmium-inducible plasmid pBCB8 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 $CdCl_2$ at 37 °C and 30 °C. The closed symbols indicate the resistance of strain COL-S$_{LGAmecA}$ to oxacillin (■) and cefoxitin (●) at 37 °C. The open symbols indicate the resistance of strain COL-S$_{LGAmecA}$ to oxacillin (□) and cefoxitin (○) at 30 °C. The triangles represent the susceptibility profile of strain COL-S to oxacillin (△) and cefoxitin (▲).

and ceftizoxine, 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-S$_{LGAmecA}$—Membrane preparations from COL and COL-S$_{LGAmecA}$ 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$_{LGA}$ 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* PBPs 1 through 3, as well as PBP2A and PBP2A$_{LGA}$. Fig. 6 demonstrates the higher affinity of membrane-anchored PBP2A for cefoxitin as compared with oxacillin, whereas the titration profile of membrane-bound PBP2A$_{LGA}$ shows a higher affinity for oxacillin as compared with cefoxitin, consistent with results obtained with the purified proteins.

PBP2A$_{LGA}$ Can Replace the Transpeptidase Function of PBP2—The bifunctional protein PBP2 is known to be essential for the

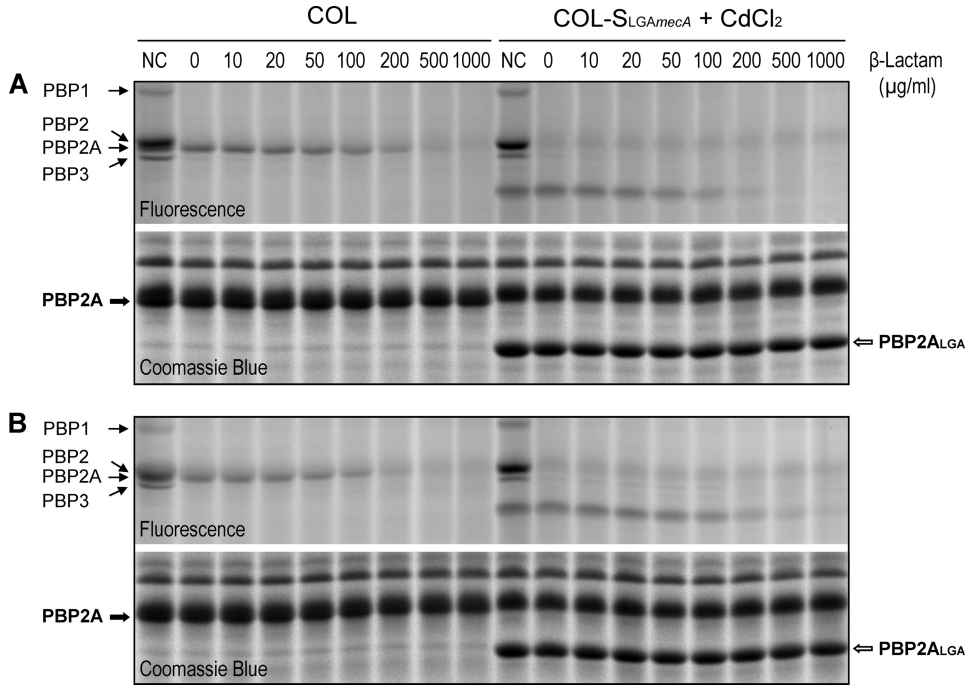

FIGURE 6. **Measurement of the affinities of PBP2A and PBP2A$_{LGA}$ from membrane preparations for two structurally different β-lactam antibiotics.** PBP2A and PBP2A$_{LGA}$ were produced in *S. aureus* COL and transductants of *S. aureus* COL-S, respectively. Membrane proteins (100 μg) prepared from COL and the transductants were analyzed by SDS-PAGE. Prior to the analysis, membrane extracts, except the ones labeled *NC*, were exposed to clavulanate (1.0 mg/ml) to make all PBPs, except for PBP2A and PBPB2A$_{LGA}$ "invisible," and then incubated with various concentrations of oxacillin (*A*) or cefoxitin (*B*). Gels were developed both by the Bocillin FL binding assay and by Coomassie Blue staining. The figure also shows the corresponding PBP patterns for the isogenic MRSA strain COL.

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 PBP2A$_{LGA}$. Fig. 7 shows that PBP2A$_{LGA}$ 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$_{/LGAmecA}$) was able to grow in the absence of IPTG, provided that the medium was supplemented with 0.2 μM cadmium chloride.

PBP2A$_{LGA}$ 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-born copy of *mecA*$_{LGA251}$:strain COL-S$_{spac}$::pbpB/$_{LGAmecA}$ was

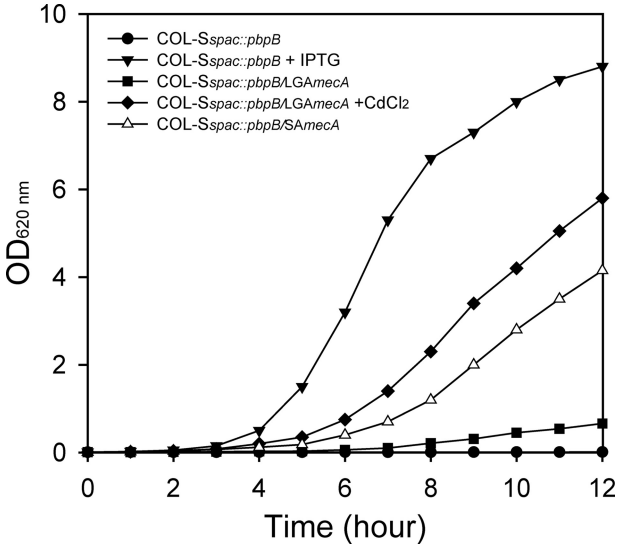

FIGURE 7. **The *mecA*$_{LGA251}$ homolog can replace the transpeptidase function of PBP2.** COL-*S$_{spac}$*::pbpB represents the conditional mutant of *S. aureus* strain of COL-S in which the transcription of *pbpB* was under the control of an IPTG-inducible promoter. COL-*S$_{spac}$*::pbpB$_{/SAmecA}$ and COL-*S$_{spac}$*::pbpB$_{/LGAmecA}$) indicate the strains to which the *mecA* gene and the *mecA* homolog were introduced by transduction, respectively. Growth of strains was followed by determination of *A* at 30 °C temperature, at which the temperature-sensitive plasmid pSTSW2C (19) carrying the *mecA* gene was stable in *S. aureus*.

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

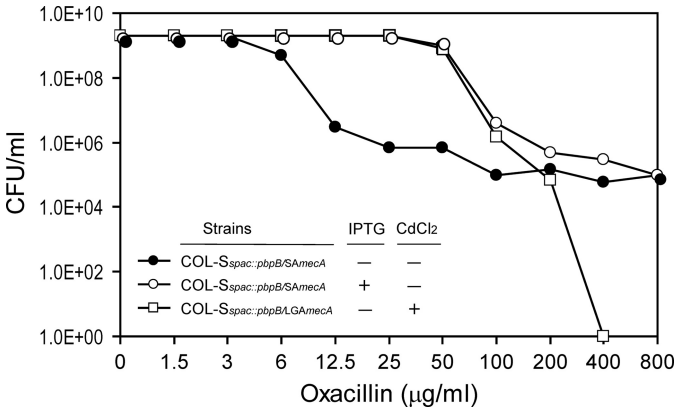

FIGURE 8. **The *mecA*LGA251 homolog does not require the TGase function of PBP2 for optimal expression of oxacillin resistance.** The number of bacteria capable of growing in the presence of different concentrations of oxacillin was determined by population analysis at 30 °C.

either with DNA probes or with one of the biochemical tests available for detection of PBP2A, the protein product of the gene. Although individual MRSA isolates were shown to vary in the structure of the chromosomal cassettes carrying *mecA*, comparison of the sequence of the *mecA* determinant in fully sequenced MRSA strains showed virtually no variation (99.9–100% identical, with one or two nucleotide substitutions). For this reason, the identification of a *mecA* structural variant in *S. aureus* LGA251, which has only 69% identity with the *mecA* genes in other MRSA strains, came as a surprise.

Initial attempts to identify the contribution of this aberrant *mecA* to the resistance phenotype of strain LGA251 were frustrating. Only traces of the transcript or a protein homolog of PBP2A were detectable in strain LGA251 under optimal condition for induction, presumably because of repression by the novel *mecI/mecR1* also carried by strain LGA251. Therefore, exploring the relationship between the variant *mecA* carried by strain LGA251 and the resistant phenotype required approaches that allowed testing the mechanism of resistance in the absence of the regulatory genes. For this, we chose two complementary approaches. In the first approach we compared biochemical and physicochemical properties of PBP2A from strain COL and PBP2A$_{LGA}$ from strain LGA251 after cloning and purification in *E. coli*. The second approach involved constructing plasmid-borne copies of the *mecA*$_{LGA251}$ free of its regulatory elements, introducing it into the background of a well characterized and drug-susceptible strain of *S. aureus*, and testing expression of the resistant phenotype by a variety of biochemical and microbiological assays.

Properties of PBP2A$_{LGA}$ Protein Cloned and Purified in E. coli—After cloning in *E. coli* followed by purification with the His tag procedure, PBP2A$_{LGA}$ was found to differ from PBP2A in a number of properties. As a first of these differences, PBP2A$_{LGA}$ showed faster mobility than PBP2A in SDS-PAGE despite the identical molecular weights of the two proteins, as determined by several methods, including mass spectrometry and Western blotting.

The mechanism of this anomaly is not clear, but is not restricted to the PBP2A$_{LGA}$ protein. A similar, abnormal pattern of mobility was also noted in the case of PBP2 of *S. aureus* and PBP4 of *Staphylococcus sciuri* (17, 21). The *S. aureus* PBP2 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 *S. sciuri*, 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$_{LGA}$ is substantially weaker than that of PBP2A, indicating incomplete denaturation of PBP2A$_{LGA}$ by SDS under the conditions of standard SDS-PAGE. These observations suggest that the aberrant mobility of PBP2A$_{LGA}$ 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$_{LGA}$ is the different temperature optimum of their activity and the thermo sensitivity of the PBP2A$_{LGA}$ protein. The CD spectra of the two proteins were shown to be similar at their respective temperature optima, but the structure of PBP2A$_{LGA}$ 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$_{LGA}$ 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$_{LGA}$ 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 *mecA*$_{LGA251}$ gene was most likely exposure to cephalosporins rather than to penicillins, which is consistent with the routine use of cephalosporins for treatment and prophylaxis of bovine mastitis in veterinary medicine[6].

Expression of mecA$_{LGA251}$ in S. aureus Strain COL-S—Introduction of plasmid-born copies of the *mecA*$_{LGA251}$ into the *S. aureus* strain COL-S allowed testing the properties of PBP2A$_{LGA}$ in direct microbiological assays. The most surprising observation was that *mecA*$_{LGA251}$ 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 *mecI/mecR1* 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 *mecA*$_{LGA251}$ (6) was retained in this genetic background.

[6] M. Holmes, personal communication.

Similar to PBP2A, PBP2A$_{LGA}$ was also able to replace the normal and essential transpeptidase function of the *S. aureus* PBP2 and support growth of a bacterial mutant in which transcription of the essential gene *pbpB* 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 glycosyltransferases (Mgts) that are known to be induced when the production of PBP2 is inhibited (34).

Expression of High-level β-Lactam Resistance in S. aureus Strain COL-S Carrying mecA$_{LGA251}$ and the TGase Function of PBP2—The two proteins PBP2A and PBP2A$_{LGA}$ 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*$_{LGA251}$, 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$_{LGA}$ 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$_{LGA}$ may "prefer" to collaborate with one of the monofunctional transglycosylases of *S. aureus* (34–36).

This preference for an alternative transglycosylase must ultimately reflect structural differences between PBP2A and PBP2A$_{LGA}$, a full understanding of which will require comparison of their crystal structures.

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