

# Contribution of Peptidoglycan Amidation to Beta-Lactam and Lysozyme Resistance in Different Genetic Lineages of *Staphylococcus aureus*

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The enzymes responsible for peptidoglycan amidation in *Staphylococcus aureus*, MurT and GatD, were recently identified and shown to be required for optimal expression of resistance to beta-lactams, bacterial growth, and resistance to lysozyme. In this study, we analyzed the impact of peptidoglycan amidation in representative strains of the most widespread clones of methicillin resistant *S. aureus* (MRSA). The inhibition of the expression of *murT-gatD* operon resulted in different phenotypes of resistance to beta-lactams and lysozyme according to the different genetic backgrounds. Further, clonal lineages CC1 and CC398 (community-acquired MRSA [CA-MRSA]) showed a stronger dependency on MurT-GatD for resistance to beta-lactams, when compared to the impact of the impairment of the cell wall step catalyzed by MurF. In the remaining backgrounds similar phenotypes of beta-lactam resistance were observed upon the impairment of both cell-wall-related genes. Therefore, for CA-related backgrounds, the predominant beta-lactam resistance mechanism seems to involve genes associated with secondary modifications of peptidoglycan. On the other hand, the lack of glutamic acid amidation had a more substantial impact on lysozyme resistance for cells of CA-MRSA backgrounds, than for hospital-acquired MRSA (HA-MRSA). However, no significant differences were found in the resistance level of the respective peptidoglycan structure, suggesting that the lysozyme resistance mechanism involves other factors. Taken together, these results suggested that the different genetic lineages of MRSA were able to develop different molecular strategies to overcome the selective pressures experienced during evolution.

## Introduction

METHICILLIN RESISTANT *Staphylococcus aureus* (MRSA) are a major cause of nosocomial infections worldwide<sup>15,18,24,27</sup> and most cases of hospital-acquired MRSA (HA-MRSA) infections are caused by a few successful multidrug-resistant epidemic clones.<sup>38</sup>

In the last two decades, the emergence of community-acquired MRSA (CA-MRSA), causing infections among healthy individuals, has been a subject of growing concern.<sup>6,7,20,33</sup> Nevertheless, nowadays, the recent changes in the epidemiology of CA-MRSA suggest that the boundaries between the hospital and community are blurring.<sup>21,29,30,50</sup>

Several studies have demonstrated that genetic backgrounds associated with CA-MRSA have a number of features that distinguish them from HA-MRSA. CA-MRSA typically have increased virulence and carry the smaller and easier to

transfer staphylococcal chromosomal cassette (SCC*mec*) type IV and V.<sup>12</sup> Interestingly, the largest SCC*mec* type II, usually found in HA-MRSA, has a significant fitness cost for the bacteria, resulting in a decrease in the growth rate, and in a reduction of toxin expression levels.<sup>9,10</sup> The balance between the virulence and antibiotic resistance costs may explain why MRSA with SCC*mec* type II are found mainly in hospital environments where high antibiotic pressure, immunocompromised individuals, and vector-mediated transmission are present.<sup>10</sup> Moreover, for CA-MRSA strains, in contrast to HA-MRSA, *mecA* was suggested not to be the primary determinant of methicillin resistance, being the expression of *pbp4* the main determinant of resistance.<sup>32</sup>

Recently, a small operon, encoding the enzymatic complex MurT-GatD, was identified to be responsible for a secondary modification of peptidoglycan in *S. aureus*, the amidation of glutamic acid in the stem peptides.<sup>17,34</sup> Inhibition of amidation

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caused reduced growth rate; reduced resistance to beta-lactam antibiotics, shown previously to be affected by auxiliary genes<sup>14</sup>; and increased sensitivity to lysozyme in HA-MRSA strain COL.<sup>17</sup>

In this communication we report that peptidoglycan amidation has different impacts in the expression of resistance to beta-lactams and to lysozyme, depending on the genetic background of the particular strain. These observations suggest that *S. aureus*, from different genetic lineages, include different elements from their core genomes in the strategies of resistance to beta-lactam and lysozyme adopted.

## Materials and Methods

### Bacterial strains and growth conditions

The 11 MRSA strains analyzed in this study are listed in Table 1. The respective mutants with *murT-gatD* and *murF* conditional mutations are listed in Table 2. *S. aureus* strains were grown at 37°C with aeration in tryptic soy broth (TSB; Difco Laboratories) or tryptic soy agar (TSA; Difco Laboratories). The strains with *murT-gatD* and *murF* conditional mutations were grown in the presence of kanamycin (50 µg/ml; Sigma) and neomycin sulfate (50 µg/ml; Sigma). Growth medium was supplemented with the appropriate concentration of cadmium chloride (CdCl<sub>2</sub>; Sigma), unless otherwise described.

### Construction of *murT-gatD* conditional mutants

The *murT-gatD* conditional mutation<sup>17</sup> was transduced, by phage 80α into the recipient strains (Table 1), as previously described,<sup>41</sup> generating the *murT-gatD* conditional mutants in different backgrounds (Table 2).

### Construction of *murF* conditional mutants

A 768-bp DNA fragment of the 5' end of *murF* gene, including the ribosome binding site but not the promoter sequence, was amplified using chromosomal DNA from strain COL as template and the specific primers PmurF'-R and PmurF'-F (Supplementary Table S1; Supplementary Data are available online at [www.liebertpub.com/mdr](http://www.liebertpub.com/mdr)). The amplified *murF* fragment and plasmid pBCB20 (R.G. Sobral and M.G. Pinho, unpublished data), carrying a CdCl<sub>2</sub> in-

ducible promoter, were digested with *SmaI* (New England Biolabs) and ligated, generating plasmid pMurF'. Plasmid pMurF' was electroporated into competent cells of RN4220 with a Gene Pulser apparatus (Bio-Rad). The correct insertion of pMurF' into RN4220 chromosome was confirmed by polymerase chain reaction, using an internal *murF* primer chosen downstream of the cloned region (PmurFdn) and an internal primer to *pCad* conditional promoter (Pcad-F) (Supplementary Table S1). The *murF* conditional mutation was then transduced, by phage 80α into the recipient strains (Table 1), as previously described,<sup>41</sup> generating the *murF* conditional mutants in different backgrounds (Table 2).

### Pulsed-field gel electrophoresis

The correct insertion of *murT-gatD* and *murF* conditional mutations into the chromosome of the recipient strains was performed by comparing the pulsed-field gel electrophoresis (PFGE) profiles of the parental strains and the respective transductants. DNA agarose disks of the parental strain and the respective mutant were prepared, digested with *SmaI*, and separated as described.<sup>8</sup>

### Southern blot analysis

*SmaI* chromosomal fragments, from the parental strain and the respective *murF* and *murT-gatD* mutants, were transferred to nylon membranes (Hybond N<sup>+</sup>; GE Healthcare) that were subsequently hybridized with specific DNA probes labeled with the ECL direct labeling system (GE Healthcare). The DNA probes used for *murT-gatD* and *murF* genes were amplified with primer pairs PmurT-D1 + PmurT-R1 and PmurF'-R + PmurF'-F, respectively (Supplementary Table S1).

### Population analysis profile

Overnight-grown cultures of the parental strains and the respective *murT-gatD* and *murF* conditional strains were plated at various dilutions on TSA plates, with increasing concentrations of oxacillin (0, 0.75, 1.5, 3, 6.25, 12.5, 25, 50, 100, 200, 400, and 800 µg/ml), and colonies were counted after incubation at 30°C for 48 hours, as previously described.<sup>13</sup>

TABLE 1. METHICILLIN RESISTANT *STAPHYLOCOCCUS AUREUS* STRAINS USED IN THIS STUDY

Strain	SCCmec type	HA/CA-MRSA	MLST (ST)	Clone	CC	Year of isolation	Country of origin	Reference
COL	I	HA-MRSA	ST250	Archaic	CC8	1965	United Kingdom	39
HDES57	IV	HA-MRSA	ST22	EMRSA15	CC22	2007	Portugal	11
HDE288	VI	HA-MRSA	ST5	Pediatric	CC5	1996	Portugal	47
HUR75	III	HA-MRSA	ST239	Brazilian	CC8	1998	Hungary	40
HUC599	II	HA-MRSA	ST5	NY/Japan	CC5	2006	Portugal	1
DEN2294	IV	CA-MRSA	ST30	Southwest-Pacific	CC30	2001	Denmark	16
ST398	V	CA-MRSA	ST398	ST398	CC398	2005	France	2
USA400	IV	CA-MRSA	ST1	USA400	CC1	1995–2003	United States	31
MW2	IV	CA-MRSA	ST1	USA400	CC1	1998	United States	6
WIS	V	CA-MRSA	ST59	Taiwan	CC59	1999	Australia	37
C377	IV	CA-MRSA	ST8	USA300	CC8	2005	Spain	46

CA-MRSA, community-acquired methicillin resistant *Staphylococcus aureus*; CC, clonal complex; HA-MRSA, hospital-acquired methicillin resistant *Staphylococcus aureus*; SCCmec, staphylococcal chromosomal cassette; ST, sequence type.

TABLE 2. MUTANT STRAINS AND PLASMIDS USED IN THIS STUDY

Strains	Relevant characteristics	Reference
<i>S. aureus</i>		
RN4220	Mc <sup>s</sup> ; restriction negative	36
M100	Mc <sup>r</sup> laboratory step mutant	51
M100p <i>CadmurT-gatD</i>	M100 with <i>murT-gatD</i> operon under <i>Pcad</i> control, Kan <sup>r</sup> , Neo <sup>r</sup>	This study
M100p <i>CadmurF</i>	M100 with <i>murF</i> gene under <i>Pcad</i> control, Kan <sup>r</sup> , Neo <sup>r</sup>	This study
COLp <i>CadmurT-gatD</i>	COL with <i>murT-gatD</i> operon under <i>Pcad</i> control, Kan <sup>r</sup> , Neo <sup>r</sup>	17
COLp <i>CadmurF</i>	COL with <i>murF</i> gene under <i>Pcad</i> control, Kan <sup>r</sup> , Neo <sup>r</sup>	This study
HUR75p <i>CadmurT-gatD</i>	HUR75 with <i>murT-gatD</i> operon under <i>Pcad</i> control, Kan <sup>r</sup> , Neo <sup>r</sup>	This study
HUR75p <i>CadmurF</i>	HUR75 with <i>murF</i> gene under <i>Pcad</i> control, Kan <sup>r</sup> , Neo <sup>r</sup>	This study
HDES57p <i>CadmurT-gatD</i>	HDES57 with <i>murT-gatD</i> operon under <i>Pcad</i> control, Kan <sup>r</sup> , Neo <sup>r</sup>	This study
HDES57p <i>CadmurF</i>	HDES57 with <i>murF</i> gene under <i>Pcad</i> control, Kan <sup>r</sup> , Neo <sup>r</sup>	This study
HAR22p <i>CadmurT-gatD</i>	HAR22 with <i>murT-gatD</i> operon under <i>Pcad</i> control, Kan <sup>r</sup> , Neo <sup>r</sup>	This study
HAR22p <i>CadmurF</i>	HAR22 with <i>murF</i> gene under <i>Pcad</i> control, Kan <sup>r</sup> , Neo <sup>r</sup>	This study
HUC599p <i>CadmurT-gatD</i>	HUC599 with <i>murT-gatD</i> operon under <i>Pcad</i> control, Kan <sup>r</sup> , Neo <sup>r</sup>	This study
HUC599p <i>CadmurF</i>	HUC599 with <i>murF</i> gene under <i>Pcad</i> control, Kan <sup>r</sup> , Neo <sup>r</sup>	This study
HDE288p <i>CadmurT-gatD</i>	HDE288 with <i>murT-gatD</i> operon under <i>Pcad</i> control, Kan <sup>r</sup> , Neo <sup>r</sup>	This study
HDE288p <i>CadmurF</i>	HDE288 with <i>murF</i> gene under <i>Pcad</i> control, Kan <sup>r</sup> , Neo <sup>r</sup>	This study
MW2p <i>CadmurT-gatD</i>	MW2 with <i>murT-gatD</i> operon under <i>Pcad</i> control, Kan <sup>r</sup> , Neo <sup>r</sup>	This study
MW2p <i>CadmurF</i>	MW2 with <i>murF</i> gene under <i>Pcad</i> control, Kan <sup>r</sup> , Neo <sup>r</sup>	This study
USA400p <i>CadmurT-gatD</i>	USA400 with <i>murT-gatD</i> operon under <i>Pcad</i> control, Kan <sup>r</sup> , Neo <sup>r</sup>	This study
USA400p <i>CadmurF</i>	USA400 with <i>murF</i> gene under <i>Pcad</i> control, Kan <sup>r</sup> , Neo <sup>r</sup>	This study
ST398p <i>CadmurT-gatD</i>	ST398 with <i>murT-gatD</i> operon under <i>Pcad</i> control, Kan <sup>r</sup> , Neo <sup>r</sup>	This study
ST398p <i>CadmurF</i>	ST398 with <i>murF</i> gene under <i>Pcad</i> control, Kan <sup>r</sup> , Neo <sup>r</sup>	This study
WISp <i>CadmurT-gatD</i>	WIS with <i>murT-gatD</i> operon under <i>Pcad</i> control, Kan <sup>r</sup> , Neo <sup>r</sup>	This study
WISp <i>CadmurF</i>	WIS with <i>murF</i> gene under <i>Pcad</i> control, Kan <sup>r</sup> , Neo <sup>r</sup>	This study
C377p <i>CadmurT-gatD</i>	C377 with <i>murT-gatD</i> operon under <i>Pcad</i> control, Kan <sup>r</sup> , Neo <sup>r</sup>	This study
C377p <i>CadmurF</i>	C377 with <i>murF</i> gene under <i>Pcad</i> control, Kan <sup>r</sup> , Neo <sup>r</sup>	This study
DEN2294p <i>CadmurT-gatD</i>	DEN2294 with <i>murT-gatD</i> operon under <i>Pcad</i> control, Kan <sup>r</sup> , Neo <sup>r</sup>	This study
DEN2294p <i>CadmurF</i>	DEN2294 with <i>murF</i> gene under <i>Pcad</i> control, Kan <sup>r</sup> , Neo <sup>r</sup>	This study
<i>Escherichia coli</i>		
DH5 $\alpha$	<i>recA endA1 gyrA96 thi-1 hsdR17 supE44 relA1 F80 DlacZDM15</i>	Invitrogen
Plasmids		
pBCB20	<i>S. aureus</i> integrative vector including <i>Pcad</i> inducible promoter, Ap <sup>r</sup> , Kan <sup>r</sup>	R.G. Sobral and M.G. Pinho, unpublished
pMurT'	pBCB20 vector with <i>murT</i> ribosome-binding site and the first 298 codons fused to <i>Pcad</i> promoter; Ap <sup>r</sup> , Kan <sup>r</sup>	17
pMurF'	pBCB20 vector with <i>murF</i> ribosome-binding site and the first 256 codons fused <i>Pcad</i> promoter; Ap <sup>r</sup> , Kan <sup>r</sup>	This study

Ap<sup>R</sup>, ampicillin resistant; Kan<sup>R</sup>, kanamycin resistant; Mc<sup>s</sup>, methicillin susceptible; Mc<sup>r</sup>, methicillin resistant; Neo<sup>R</sup>, neomycin resistant.

#### Peptidoglycan isolation for lysozyme lytic assays

Isolation of cell wall was performed as described previously.<sup>3</sup> Briefly, cells were harvested by centrifugation, washed twice with cold 0.9% NaCl, and boiled for 20 minutes. After chilling, the cells were washed twice and disrupted using 106-mm glass beads (Sigma) and FastPrep FP120 apparatus (Bio 101). The suspension was then washed, and boiled for 30 minutes in 5% sodium dodecyl sulfate and 50 mM Tris-HCl (pH 7), to remove noncovalently bound proteins. After centrifugation, the cell wall fragments were diluted in 0.1 M Tris-HCl (pH 6.8) and incubated with 0.5 mg/ml trypsin for 16 hours at 37°C to degrade cell-bound proteins. Purified cell walls were washed with water and lyophilized and treated with 49% hydrofluoric acid for 48 hours at 4°C, to remove teichoic acids. The purified peptidoglycan was washed with water and lyophilized.

#### Turbidometric assay of peptidoglycan hydrolysis

To analyze the susceptibility of peptidoglycan to lysozyme hydrolysis, a turbidometric assay was performed as described previously.<sup>3,17,19</sup> Briefly, purified peptidoglycan was sonicated in 100 mM sodium-potassium phosphate buffer (pH 6.6). Egg white lysozyme (Sigma) was added (300  $\mu$ g/ml) and the reaction was incubated at 37°C. The optical density was monitored at 595 nm in 96-well microplates (pure grade, Brand) using a microplate reader (Infinite F200 Pro; Tecan).

#### Determination of lysozyme resistance of *S. aureus* growing cells

The impact of lysozyme on exponentially growing cultures was determined as described previously.<sup>17,19</sup> Overnight cultures

of the conditional mutants, grown with inducer, were inoculated into fresh TSB, with and without inducer. The cultures were incubated at 37°C to an OD<sub>620nm</sub> of 1.0. Then, each culture was diluted 1:10 into fresh TSB, with and without inducer, and lysozyme (300 µg/ml) was added as the OD<sub>620nm</sub> reached 1.0. The growth was monitored for several hours.

#### Statistical analysis

A two-tailed Student's *t* test with Welch correction was used to determine the significance of differences in lysozyme digestion within groups of CA-MRSA and HA-MRSA. Differences were considered statistically significant when *p* was <0.005. The Graph Pad Prism 5.0 package was used.

## Results

The impact of the impairment of peptidoglycan amidation on oxacillin resistance was previously demonstrated by the construction and characterization of the *murT-gatD* conditional mutation in strain COL. This mutation was shown to impact not only beta-lactam resistance, but also growth rate and lysozyme resistance.<sup>17</sup> In this study, we first observed that the effects of *murT-gatD* inhibition in the resistance level of strain COL and strain MW2 were clearly distinct, as observed through oxacillin inhibition halos (1-mg disc) (Supplementary Fig. S1). The decrease in the resistance level of the strain was significantly more pronounced for MW2*pCadmurT-gatD*, showing a twofold wider inhibition halo. To test that this behavior was the result of different genetic backgrounds and not a strain-specific trait, the mutation was transferred to another strain of the same clone USA400 and a similar resistance profile was obtained (Fig. 1A). These results led to the hypothesis that *murT-gatD* expression and/or the enzymatic step catalyzed by the MurT-GatD complex could have different physiological consequences depending on the genetic background.

To address this hypothesis, the *murT-gatD* conditional mutation was transduced to representative strains of the most widespread MRSA clones, among both HA-MRSA and CA-MRSA (Table 1).

The correct transfer of the mutation was determined by comparing the *Sma*I PFGE profiles of the parental strains and the respective transductants (Supplementary Fig. S2). In addition, Southern blot analysis using specific probes for *murT-gatD* genes confirmed the correct insertion of the conditional mutation.

#### Impact of *murT-gatD* conditional mutation on resistance to beta-lactam antibiotics in different MRSA genetic backgrounds

The impact of *murT-gatD* conditional mutation on the beta-lactam resistance level of the different MRSA strains was evaluated by performing oxacillin population analysis profiles.

The most striking observation was that MW2/USA400, ST398, and WIS CA-MRSA *murT-gatD* conditional mutants (Fig. 1A) grown in the absence of inducer were overall less resistant to oxacillin, when compared with the HA-MRSA mutant strains (Fig. 1B). A first early drop in the number of cfu/ml, occurring at 0.75 µg/ml, was common to

all analyzed mutant strains, followed by a high frequency of resistant subpopulations, able to grow on higher concentrations of antibiotic. Strikingly, while for the HA-MRSA (COL, HUR75, and HDES57) the subpopulations of the mutants were able to grow on antibiotic concentrations near the MIC (minimal inhibitory concentration) of the parental strain (from 50 to 800 µg/ml, Fig. 1B), for the CA-MRSA (MW2, USA400, ST398, and WIS), the mutants' subpopulations only grew at low antibiotic concentrations (from 0.75 to 6.25 µg/ml, Fig. 1A). Consequently, complete growth inhibition occurred at much lower antibiotic concentrations for *murT-gatD* mutants of CA-MRSA backgrounds (Fig. 1A).

Within the CA-MRSA strains, C377*pCadmurT-gatD* and DEN2294*pCadmurT-gatD* showed a less striking decrease in resistance to oxacillin (Fig. 2A), with subpopulations that were able to grow up to 100 µg/ml. In fact, C377*pCadmurT-gatD* showed an overall resistance profile similar to HUR75 *pcadmurT-gatD* (Figs. 2A and 1B, respectively). The similarities between these resistance profiles were consistent with the fact that HUR75 and C377 are genetically related, belonging to the same clonal complex (CC8).

Likewise, DEN2294 (ST30-IV, Southwest Pacific clone) is genetically related to the HA-MRSA ST36-MRSA-II (EMRSA-16) as they are descendants from the common ancestral ST30-MSSA.<sup>45</sup> A conditional *murT-gatD* mutant was not constructed in the ST36-MRSA-II background, as all the strains available were resistant to the selectable marker of the pMurT' integrative plasmid (kanamycin).

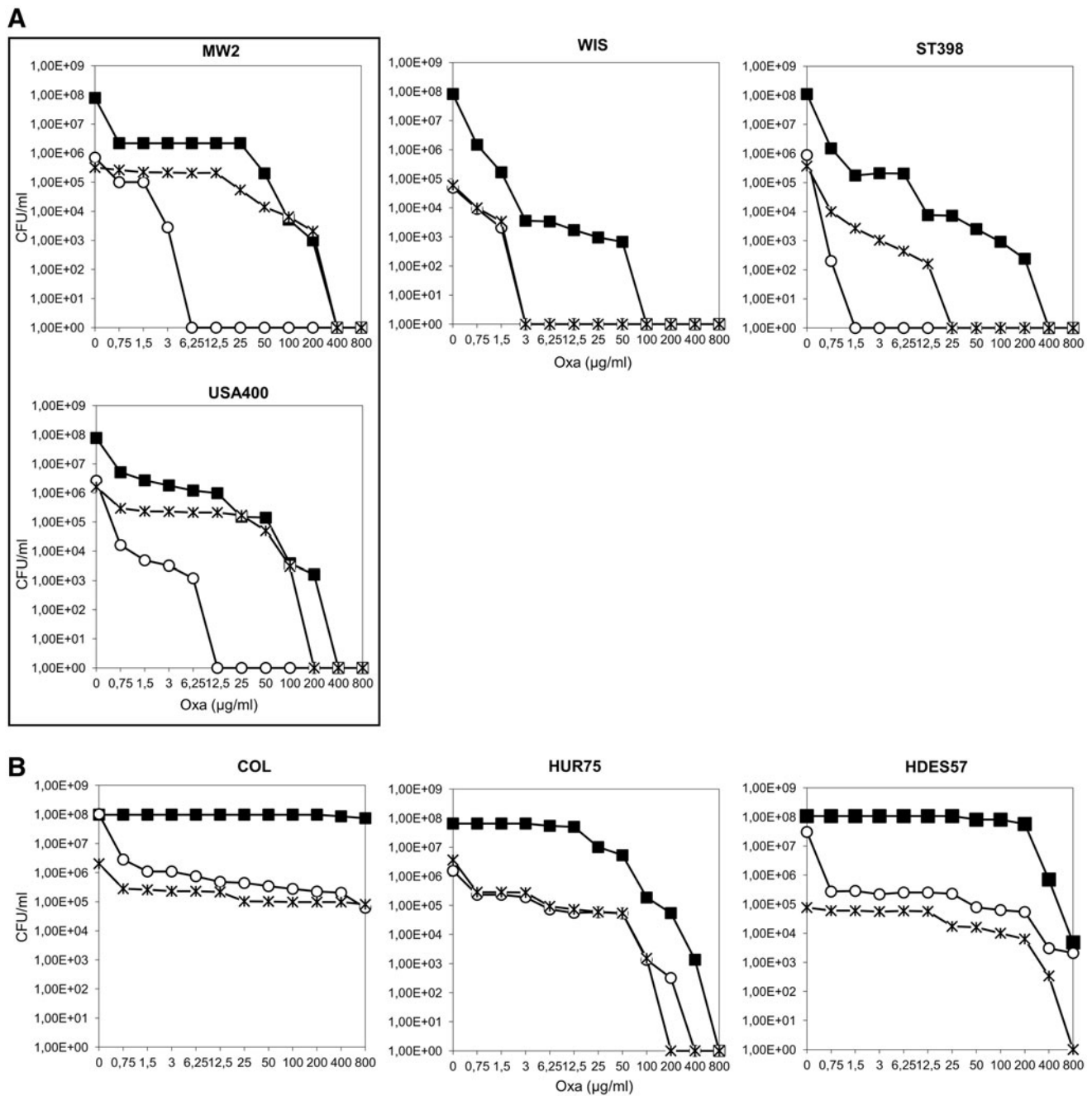
Regarding the Pediatric and New York/Japan clones (HDE288 and HUC599, respectively) the parental strains exhibited, together with a high frequency of resistant subpopulations, lower MIC values (0.75 µg/ml) than the previously analyzed HA-MRSA strains (50–800 µg/ml, Fig. 2B). Consistently, for these strains, the *murT-gatD* mutation had a complete inhibitory impact at lower antibiotic concentrations (6.25 and 100 µg/ml for HDE288 and HUC599 mutants, respectively, Fig. 2B), than the remaining HA-MRSA mutants.

To explore whether this behavior is associated with distinct steps of peptidoglycan biosynthesis, the impact of a *murF* conditional mutation was also studied in the same genetic backgrounds.

#### Impact of *murF* conditional mutation on resistance to beta-lactam antibiotics in different MRSA genetic backgrounds

A conditional mutation for *murF* gene (*pCadmurF*) was constructed using the same *pCad* inducible promoter, transduced from RN4220*pCadmurF* to the strains listed in Table 1, and oxacillin population analysis profiles were performed (Figs. 1 and 2). In the absence of inducer, the *murF* conditional mutants were impaired in the last biosynthetic cytoplasmic step, catalyzed by MurF protein, the addition of the D-alanyl-D-alanine terminus to the stem peptide.<sup>48</sup>

For most clonal lineages the level of resistance to oxacillin was similar for *murT-gatD* and *murF* mutants (Figs. 1B and 2B). However, for MW2/USA400 and ST398 CA-MRSA strains, all CA-MRSA strains, the inhibition of *murT-gatD* transcription caused a more pronounced effect on oxacillin resistance, than inhibition of *murF* transcription (Fig. 1A).

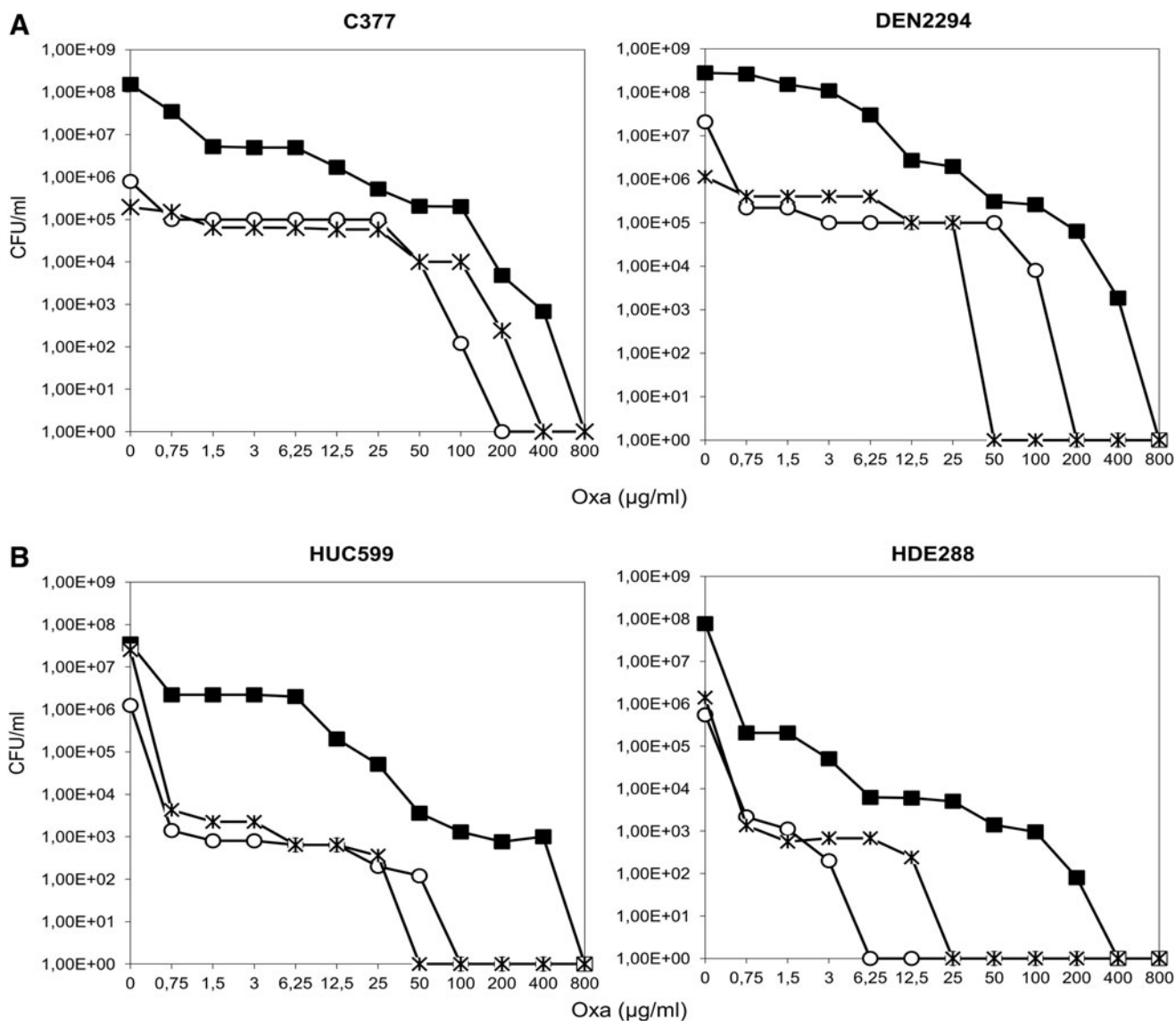


**FIG. 1.** Impact of *murT-gatD* and *murF* conditional mutations on the oxacillin resistance profiles of MW2, USA400, WIS, ST398, COL, HUR75, and HDES57 strains. Overnight cultures of the parental strains and conditional mutants grown with  $\text{CdCl}_2$  inducer were plated on TSA containing increasing concentrations of oxacillin. Plates were incubated for 48 hours at  $30^\circ\text{C}$ . (■) Oxacillin population analysis profile of parental strains; (○) oxacillin population analysis profile of *murT-gatD* conditional mutants; (✱) oxacillin population analysis profile of *murF* conditional mutants. Oxacillin population analysis profile of (A) CA-MRSA strains and (B) HA-MRSA strains. CA-MRSA, community-acquired methicillin resistant *Staphylococcus aureus*;  $\text{CdCl}_2$ , cadmium chloride; HA-MRSA, hospital-acquired MRSA; TSA, tryptic soy agar.

*Impact of murT-gatD and murF conditional mutations on beta-lactam resistance in a mecA-negative strain resistant to methicillin*

The *pCadmurT-gatD* and *pCadmurF* conditional mutations were transduced to the M100 strain (Table 2), a laboratory step mutant selected for methicillin resistance,<sup>51</sup> which encodes a modified PBP3,<sup>43</sup> and does not contain

*mecA*. The inhibition of *murF* transcription, in the background of M100 strain, resulted in a decrease in cell viability, shown by a drop in the number of cfu/ml from  $10^8$  to  $10^6$  (Fig. 3). However, no effect was observed in the oxacillin resistance level. In contrast, the impairment of *murT-gatD* caused, besides the same decrease in viability, a fourfold decrease in oxacillin resistance; the conditional mutant, grown in the absence of inducer, showed complete



**FIG. 2.** Impact of *murT-gatD* and *murF* conditional mutations on the oxacillin resistance profiles of C377, DEN2294, HUC599, and HDE288 strains. Overnight cultures of the parental strains and conditional mutants, grown with  $\text{CdCl}_2$  inducer, were plated on TSA containing increasing concentrations of oxacillin. Plates were incubated for 48 hours at  $30^\circ\text{C}$ . (■) Oxacillin population analysis profile of parental strains; (○) oxacillin population analysis profile of *murT-gatD* conditional mutants; (✱) oxacillin population analysis profile of *murF* conditional mutants. Oxacillin population analysis profile of (A) CA-MRSA strains and (B) HA-MRSA strains.

growth inhibition at  $2\ \mu\text{g/ml}$  of oxacillin, in contrast to the parental strain ( $8\ \mu\text{g/ml}$ ) (Fig. 3).

#### Impact of *murT-gatD* conditional mutation on lysozyme resistance in different MRSA genetic backgrounds

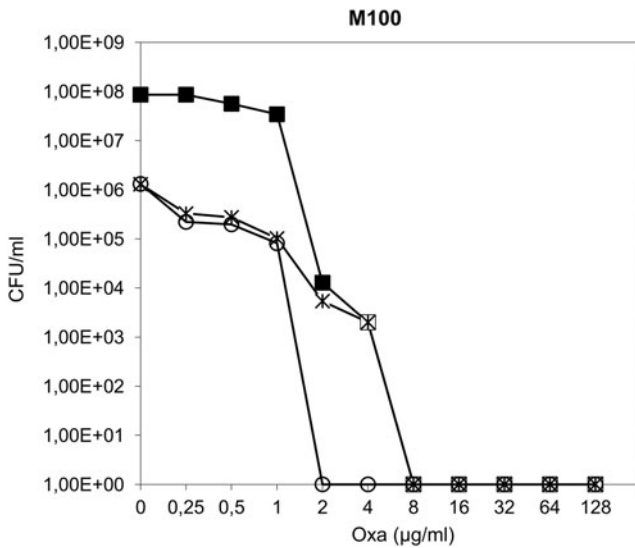
**Lysozyme resistance assays in living cells.** To evaluate the impact of *murT-gatD* conditional mutation on *S. aureus* intrinsic lysozyme resistance, in the several genetic backgrounds, the *murT-gatD* conditional mutants were grown, in the absence and in the presence of inducer, and treated with muramidase during the exponential phase. The cell density of the cultures was then monitored for several hours.

The parental strains, as the conditional mutants grown with inducer, showed no growth alteration upon addition of

lysozyme to the medium (shown for HDES57, HDE288, MW2, and C377 strains and for the respective mutants, grown without inducer, Fig. 4; data not shown for the remaining strains), confirming that all these strains are resistant.

To address the effects of the impairment of *murT-gatD* transcription on lysozyme resistance level, the cell density values of each mutant culture, grown with and without lysozyme, were compared at 90 minutes after the addition of muramidase (Fig. 5A).

Overall, *murT-gatD* mutants constructed in CA-MRSA backgrounds were more sensitive to lysozyme, when compared with mutants constructed in HA-MRSA backgrounds. In fact, while all CA-MRSA mutants showed a decrease in optical density above 70% (mean value of  $86.1\% \pm 9.6\%$ ) when grown in the presence of lysozyme, in HA-MRSA the



**FIG. 3.** Impact of *murT-gatD* and *murF* conditional mutations on the oxacillin resistance profile of *mecA*-negative M100 strain. Overnight cultures of the parental strain and conditional mutants, grown with CdCl<sub>2</sub> inducer, were plated on TSA containing increasing concentrations of oxacillin. Plates were incubated for 48 hours at 30°C. (■) Oxacillin population analysis profile of M100 parental strain; (○) oxacillin population analysis profile of *murT-gatD* conditional mutant; (\*) oxacillin population analysis profile of *murF* conditional mutant.

decrease was much more variable, ranging between 23.1% and 90.6% (Fig. 5A). The difference in the mean lysozyme digestion level, between groups of CA-MRSA and HA-MRSA, was statistically significant ( $p < 0.005$ , Student's *t* test). Interestingly, mutants DEN2294p*CadmurT-gatD* and C377p*CadmurT-gatD* were more resistant to lysozyme, than the remaining CA-MRSA strains, showing again a different behavior, as previously observed for the oxacillin resistance profiles (Figs. 5A and 2A).

#### Lysozyme resistance assays with purified peptidoglycan

To determine whether the mutant phenotypes, observed *in vivo*, were directly associated with the lack of amidation of peptidoglycan, or whether they were associated with other strain specificities, the peptidoglycan of the parental strains and the respective mutant, grown without inducer, was isolated and purified. The peptidoglycan concentration was adjusted and after addition of lysozyme, the optical density was monitored to assess the amount of peptidoglycan digested.

The results of the lytic assays showed no statistically significant differences (Student's *t* test) between the lysozyme resistance of the purified peptidoglycan of the different mutant strains (Fig. 5B).

The comparison between the susceptibility to lysozyme of *murT-gatD* mutants' living cells and their respective purified peptidoglycan (Fig. 5A, B) showed no correlation. In fact, while cells from CA-MRSA mutants were more susceptible to lysozyme than cells from HA-MRSA mutants, their respective purified peptidoglycan showed no significant variability between the resistance levels.

## Discussion

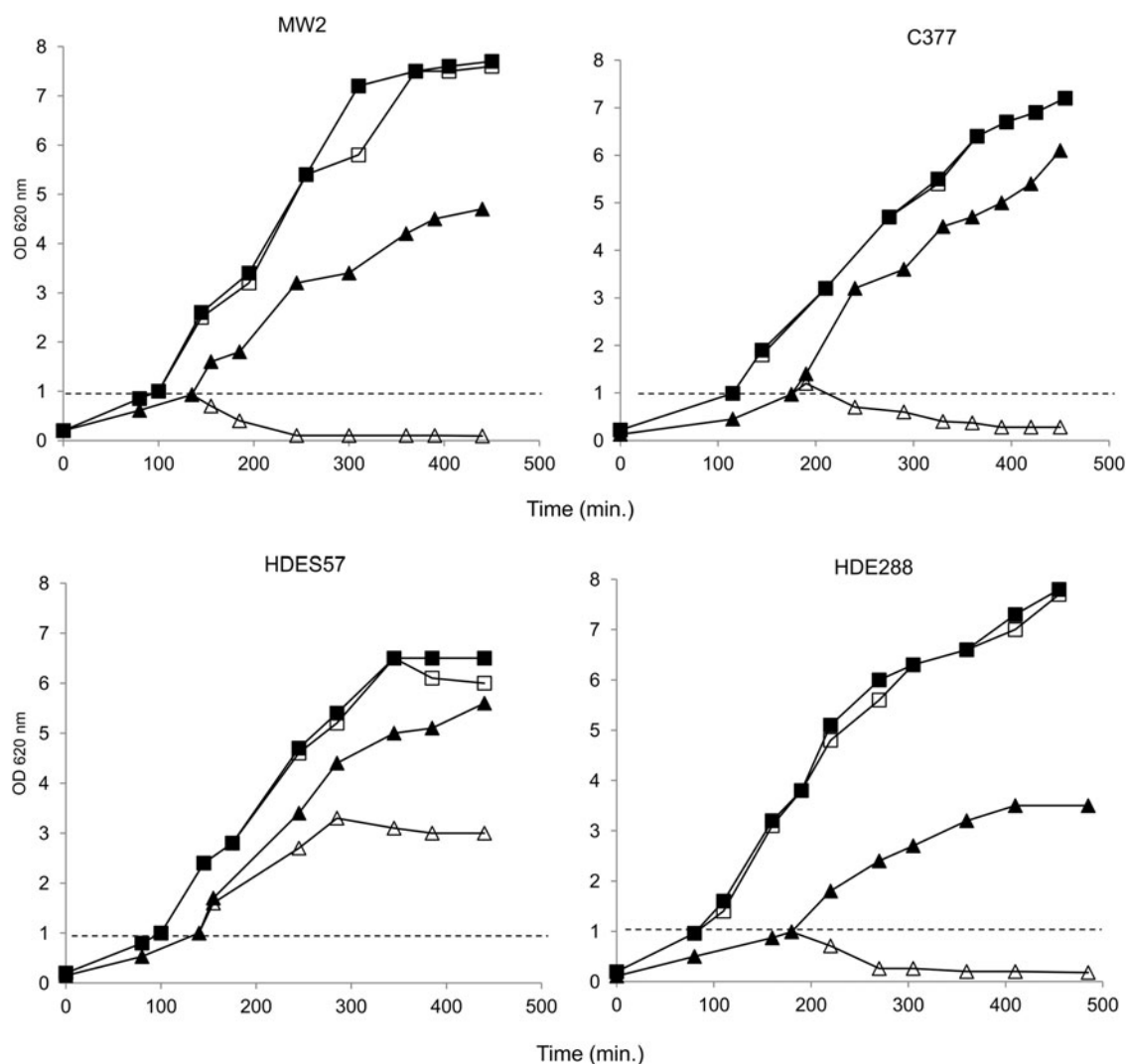
In the last half-century, the cell wall biosynthetic pathway has been extensively studied, namely, for its role as an antimicrobial target. However, the enzymes responsible for the amidation of D-glutamic acid of staphylococcal peptidoglycan were described only recently. Figueiredo *et al.*<sup>17</sup> identified, in MRSA strain COL, the *murT-gatD* operon whose protein products catalyze the amidation of peptidoglycan and showed that this cell wall modification is important for optimal growth, beta-lactam resistance, and sensitivity to the host defense factor lysozyme. Munch *et al.*<sup>34</sup> demonstrated that both enzymes, MurT and GatD, are essential for survival and interact as a glutamine amidotransferase bi-enzymatic complex.

Several genes of the peptidoglycan biosynthetic pathway, among other housekeeping genes, are involved in the beta-lactam resistance mechanism. In fact, the mechanism of resistance of MRSA strains is not simply *mecA*-dependent, but also needs the optimal expression of the so-called auxiliary genes<sup>4,14,25,35</sup> among which are essential cell-wall-related determinants, such as *murE*,<sup>28</sup> *murF*,<sup>49</sup> *pbp2*,<sup>44</sup> *pbp1*,<sup>42</sup> *femABX*,<sup>5</sup> and *murT-gatD* operon.<sup>17</sup> However, the impact of different steps of peptidoglycan synthesis in beta-lactam resistance of MRSA strains from different genetic backgrounds, was only assessed for PBP4.<sup>32</sup> Previously, Katayama *et al.*<sup>23</sup> showed that impairment of *pbp4* does not affect the beta-lactam resistance level of MRSA, being not classified as an auxiliary gene. Later, Memmi *et al.*<sup>32</sup> showed that *pbp4* is an auxiliary gene in CA-MRSA, being the key player in the resistance mechanism of these specific strains.

In this communication, the *murT-gatD* conditional mutation was studied in the background of the major contemporary MRSA clones. Different impacts in the oxacillin resistance profile were observed for *murT-gatD* depletion in several MRSA genetic backgrounds, with a more pronounced effect on CA-MRSA-related backgrounds, when compared with HA-MRSA. The conditional mutants of MW2/USA400, ST398, and WIS strains showed complete growth inhibition at antibiotic concentrations ~100-fold lower than HA-MRSA mutants. However, this effect was not shared by all CA-MRSA strains; the conditional mutants of C377 and DEN2294, belonging to lineages highly disseminated in community settings (USA300 and Southwest Pacific clones, respectively), showed a less striking decrease in resistance to oxacillin. In the case of C377, this behavior could be explained by the fact that this strain is genetically related with the HA-MRSA HUR75 strain, as they belong to the same clonal complex (CC8). Likewise, DEN2294 CA-MRSA (ST30-IV, Southwest Pacific clone) has a genetic background related to the hospital-acquired ST36-MRSA-II (EMRSA-16) as DEN2294 and ST36-MRSA-II have a common ancestral, ST30-MSSA.<sup>22,45</sup>

Although the differences in oxacillin resistance decrease observed between the CA- and HA-related strains are clear, the molecular mechanism behind these different phenotypes is probably directly associated rather with the strains' clonal complexes, and therefore, with their genetic background.

An association between the strains' genetic background and their capacity to acquire and maintain a recombinant plasmid expressing *mecA* was previously observed.<sup>22</sup> Strains



**FIG. 4.** Impact of *murT-gatD* conditional mutation on lysozyme resistance in HDES57, HDE288, MW2, and C377 strains and the respective mutants, grown without inducer. Overnight cultures were diluted to  $OD_{620nm}$  0.1 and were incubated at 37°C to  $OD_{620nm}$  of 1.0. Then, each culture was diluted into fresh medium and lysozyme (300  $\mu$ g/ml) was added at  $OD_{620nm}$  1.0, as indicated by the dashed line. Conditional mutant grown without inducer, in the presence ( $\Delta$ ) and in the absence of lysozyme ( $\blacktriangle$ ); parental strain grown in the presence ( $\square$ ) and in the absence of lysozyme ( $\blacksquare$ ).

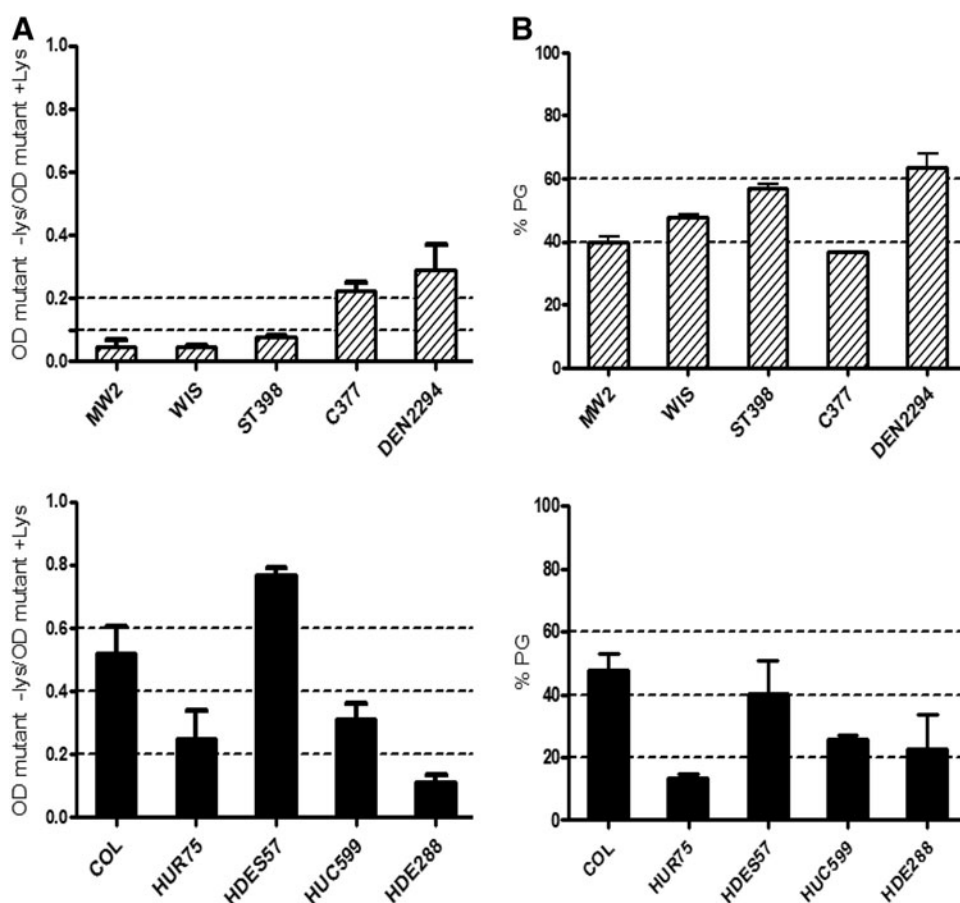
from clonal complexes CC1 and CC5 were, among the major MRSA lineages tested, the ones that were less efficiently transformed. However, CC5 lineage has been recently described to be well adapted to the hospital environment through the efficient acquisition of resistance to new antibiotics,<sup>26</sup> suggesting that strains belonging to CC5 are able to easily acquire *mecA*, but not able to efficiently maintain it. Therefore, the CC5 lineage seems to be less dependent on the presence of *mecA* than other major lineages, for the efficient expression of resistance to beta-lactams. One hypothesis is that in strains of CC5 beta-lactam resistance relies on the presence of specific housekeeping genes, namely, *murT-gatD*, although the presence of *mecA* would still be essential.

Coincidentally, strains belonging to CC1 (MW2/USA400) typically associated with the community onset, and CC5 (HDE288 and HUC599), which includes hospital-related strains, were among the ones that showed higher impact

from *murT-gatD* impairment. The other strains that showed the same level of impact were WIS and ST398 (CC59 and CC398, respectively), which harbor the small SCC<sub>mec</sub> type V, suggesting that their genetic background would also not favor the stability of *mecA* expression.

In this line of thought, the clonal complexes CC8, CC22, and CC30, which showed higher efficiency of transformation with *mecA* and stability of *mecA* expression,<sup>22</sup> were, in our study, represented by the strains showing less impact of *murT-gatD* impairment (COL, HUR75, C377, HDES57, and DEN2294). Taken together, these observations suggest that the genetic backgrounds less prone to receiving *mecA* gene recruited preferentially specific housekeeping genes, such as *murT-gatD*, for their beta-lactam resistance strategy. To address the importance of *mecA* presence in this alternative resistance strategy, *murT-gatD* mutation was transferred into a *mecA*-independent resistant strain, M100, with a truncated PBP3. The *murT-gatD* impairment resulted in a





**FIG. 5.** Impact of *murT-gatD* conditional mutation on lysozyme resistance. (A) Effect of lysozyme (300  $\mu$ g/ml) on the growth rate of the conditional mutants grown without inducer. The impact of lysozyme is represented as the ratio between the optical density of the culture of the mutant grown with lysozyme (+lys) and without lysozyme (–lys), 90 minutes after addition of the muramidase. (B) Effect of lysozyme (300  $\mu$ g/ml) on purified PG from the conditional mutants grown without inducer. The impact of lysozyme is represented as the percentage of undigested peptidoglycan, 40 minutes after addition of the muramidase. Dashed bars, conditional mutants of CA-MRSA strains; solid bars, conditional mutants of HA-MRSA strains. Also represented are the mean and standard deviation of triplicate experiments. PG, peptidoglycan.

decrease in the level of resistance of the M100 strain, indicating that peptidoglycan amidation is essential for a *mecA*-independent resistant strategy.

To assess the importance of different steps of peptidoglycan biosynthesis in this alternative strategy of *mecA*-associated resistance, *murF* gene was chosen for further testing. On one hand, MurF catalyzes a crucial step of the primary pathway of peptidoglycan biosynthesis; on the other hand, it is a well-documented auxiliary gene for COL background.<sup>48</sup> While for most genetic backgrounds the impact of *murF* impairment in the resistance profile was comparable to the one of *murT-gatD*, for MW2/USA400 and ST398 strains, *murT-gatD* conditional mutation showed a drastic and unique effect. Further, the inhibition of *murF* transcription did not affect the level of resistance in the *mecA*-negative strain M100, suggesting that the contribution of *murF* auxiliary gene for beta-lactam resistance is related to the presence of *mecA*.

Therefore, the alternative strategy for beta-lactam resistance seems to rely on genes involved in peptidoglycan secondary modifications, as secondary cross-linking (*pbp4*) and amidation (*murT-gatD*).<sup>32</sup> Recently, Zapun *et al.*<sup>52</sup>

showed that in *Streptococcus pneumoniae*, peptidoglycan amidation catalyzed by MurT-GatD complex is necessary for efficient cross-linking by PBP2a, PBP2b, and PBP2 $\times$ . PBP1a retained some activity for nonamidated lipid precursors. Although the substrate preferences of *S. aureus* PBPs regarding the amidation status of the precursor molecule are not known, it seems reasonable to speculate that PBP4 and/or PBP2 also require amidated precursors to perform transpeptidation, as these two proteins appear to be involved in the alternative mechanism of resistance to beta-lactams.<sup>32</sup>

Moreover, besides being essential for optimal beta-lactam resistance, the *murT-gatD* operon is also needed for optimal lysozyme resistance, evidencing its role in virulence. We also observed that the impairment of *murT-gatD* operon had a strong impact on lysozyme resistance in CA-MRSA backgrounds. For the HA backgrounds, the impact of this mutation is more variable, according to the genetic background. However, the lysozyme resistance levels of purified peptidoglycan were similar for strains from both CA and HA settings. This observation indicates that specific factors, intrinsic to the strain genetic background, contribute to the

final lysozyme resistance level, although dependent on the amidation status of the cell wall.

The results reported in this communication suggest that peptidoglycan amidation is involved through different mechanistic links in the beta-lactam resistance strategies of strains from distinct backgrounds, evidencing in this way the existence of more than one physiological approach for survival to antibiotic stress.

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### Disclosure Statement

No competing financial interests exist.

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