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Inhibition of D-alanylation of teichoic acids overcomes resistance of methicillin-resistant *Staphylococcus aureus*

Delphine Coupri[1], Nicolas Verneuil[1], Axel Hartke[1], Axelle Liebaut[1], Thierry Lequeux[2], Emmanuel Pfund[2] and Aurélie Budin-Verneuil[1]*

[1]*Normandie Univ, UNICAEN, U2RM, 14000 Caen, France;* [2]*Normandie Université, Laboratoire de Chimie Moléculaire et Thioorganique UMR 6507, ENSICAEN, UNICAEN, CNRS, 6 Bd. du Maréchal Juin, 14050 Caen, France*

*Corresponding author. E-mail: aurelie.verneuil@unicaen.fr

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Background: MRSA are high-priority multidrug-resistant pathogens. Although there are still some antibiotics active against MRSA, continuous efforts to discover new antibiotics and treatment strategies are needed because resistance to these new drugs has already been reported.

Objectives: Here we explore if D-alanylation of teichoic acids (TAs) mediated by the *dlt* operon gene products might be a druggable target to overcome β-lactam-resistance of MRSA.

Methods: MICs and bactericidal effects of several β-lactam antibiotics were monitored in a panel of clinical MRSA strains with genetic or chemically induced deficiency in D-alanylation of TAs. Efficiency of the chemical inhibitor to rescue MRSA-infected larvae of *Galleria mellonella* as well as its ability to prevent or eradicate biofilms of *S. aureus* were analysed.

Results: Genetic inactivation of the Dlt system or its chemical inhibition re-sensitizes MRSA to β-lactams. Among the 13 strains, the most pronounced effect was obtained using the inhibitor with imipenem, reducing the median MIC from 16 to 0.25 mg/L. This combination was also bactericidal in some strains and significantly protected *G. mellonella* larvae from lethal MRSA infections. Finally, inactivation of D-alanylation potentiated the effect of imipenem on inhibition and/or eradication of biofilm.

Conclusions: Our combined results show that highly efficient inhibitors of D-alanylation of TAs targeting enzymes of the Dlt system should be promising therapeutic adjuvants, especially in combination with carbapenems, for restoring the therapeutic efficacy of this class of antibiotics against MRSA.

Introduction

Antibiotic resistance is a major global health problem increasing the number of deaths and the societal costs of infections.[1] To counteract this crisis, the WHO has published a priority list of MDR microorganisms for which research and development of new antibiotics or therapeutic strategies is urgently needed.[2] Methicillin-resistant *Staphylococcus aureus* (MRSA) are classified to be of high priority among the listed drug-resistant pathogens. Treatment options are limited due to resistance to almost all β-lactams, except for some last-generation cephalosporins. However, resistance to these last drugs in clinical MRSA isolates from patients has already been reported.[3] β-Lactams target penicillin binding proteins (PBPs) by inhibiting their transpeptidase activity, thereby blocking cell wall biosynthesis and leading finally to cell death.[4–6] In most clinical isolates, resistance to β-lactams is due to an inducible, acquired PBP, PBP2a. This protein is encoded by the *mecA* gene or its homologues (*mecB, mecC*, and *mecD*).[7] The corresponding genes are carried on genetically diverse but related SCCmec elements (where SCC stands for staphylococcal cassette chromosome).[7] PBP2a has low affinity for β-lactams and therefore can substitute the transpeptidase activities of the native staphylococcal PBPs allowing cell wall synthesis in the presence of the drugs. However, many other *S. aureus* core genes are necessary for PBP2a-mediated resistance.[3] For example, genetic or biochemical inactivation of the first step of wall teichoic acid (WTA) biosynthesis (TarO) rendered MRSA highly susceptible to β-lactam antibiotics.[8] WTAs and lipoteichoic acids (LTAs) are negatively charged cell wall polymers of Gram-positive bacteria, tagged with D-alanine esters, which confer a positive charge on these structures.[9] D-Alanine incorporation into WTAs requires four proteins encoded by the *dltABCD* operon which are all essential for the process.[10] Dlt

mutants demonstrated an increase in the susceptibility of bacteria to cationic antimicrobial peptides[9] as well as cationic antibiotics.[11] However, the MICs of different β-lactam antibiotics, which are neutral or negatively charged molecules, were only marginally affected in a D-alanylation-deficient MRSA strain, ranging from slightly increased or decreased MICs.[11,12] On the other hand, it has been previously shown that growth of *Bacillus subtilis* was blocked in presence of the cationic glycopeptide vancomycin and a chemical inhibitor that shows high affinity for DltA of *B. subtilis in vitro*.[13] We recently showed that genetic inactivation or pharmacological inhibition of D-alanylation increased the bactericidal activity of β-lactam combination treatments against pathogenic enterococci.[14] Therefore, the initial objective of the present work was to study if D-alanylation-deficient strains of MRSA would also be more susceptible to killing by β-lactam antibiotics. This was indeed the case, but we also found that D-alanylation-deficient strains demonstrated highly decreased MICs when treated with β-lactams in monotherapy. Furthermore, we show here that these treatments also affect biofilm formation and, in the case of genetic D-alanylation deficiency, biofilm eradication. Finally, we show that inhibition of D-alanylation increased survival of infected insect larvae of *Galleria mellonella* treated with β-lactam antibiotics, arguing that inhibition of D-alanylation is a realistic highly promising target to overcome MRSA β-lactam resistance.

Materials and methods

Bacterial strains, media and reagents

Bacterial strains used in this study are listed in Table 1. Cultures were grown in Brain Heart Infusion (BHI) (Biokar diagnostics, France) for physiological assays or in Mueller-Hinton (MH) (Biokar diagnostics) for mutagenesis assays. Vancomycin, amoxicillin, cefotaxime, imipenem, penicillin G and chloramphenicol were purchased from Sigma–Aldrich (MO, USA) and oxacillin was purchased from VWR (PA, USA). Lyophilized DltA inhibitor {5′-O-[N-(D-alanyl)-sulfamoyl]-adenosine} was synthesized as described by May *et al.*[13] and solubilized at 10 mM in sterilized pure water.

Mutagenesis and complementation of dltA gene

Construction of Δ*dltA* mutants in the parental strains MW2 and CNRI of *S. aureus* was performed with plasmid pMADΔ*dltA* (Table 1).[12] After passaging in *S. aureus* RN4220,[15] the pMADΔ*dltA* was isolated and introduced in *S. aureus* MW2 or CNRI by electroporation. Deletion was done essentially as previously described.[12] The truncation of the *dltA* gene was confirmed by PCR and sequencing.

Complementation of the Δ*dltA* mutant strains in *S. aureus* was performed as previously described by using the vector pRB473 carrying the entire wild-type *dlt* operon (Table 1).[16]

Determination of growth kinetics

96-Well microplates (Starlab, France) containing 200 μL of fresh BHI medium were inoculated with overnight cultures to an OD_{600} of 0.02 with DltA inhibitor (1 mM) when needed. Plates were incubated at 37°C with shaking (orbital amplitude of 3 mm) in a Tecan microplate reader (Infinite M Nano). OD_{600nm} was measured every 10 min for 24 h.

MIC determination and bacterial survival

Cultures in early-log phase prepared in fresh BHI or MH from an overnight culture were diluted to an OD_{600} of 0.05 and incubated at 37°C with shaking (120 rpm) until an OD_{600} of 0.5 was reached. The MIC was determined according to the CLSI method. The DltA inhibitor was added when

Table 1. *Staphylococcus aureus* strains and plasmids used in this study

Common name	Characteristics	Origin/reference
Strains		
MW2	CA-MRSA, WT strain	30
MW2Δ*dltA*	MW2 strain deficient in *dltA* gene	This study
MW2Δ*dltA*comp	MW2Δ*dltA* complemented with pRB473-*dlt*	This study
CHU1	HA-MRSA, T-SAR-01	University Hospital Center of Caen
CHU17	HA-MRSA, T-SAR-17	University Hospital Center of Caen
CNRI	HA-MRSA, SCCmec type I, HT20020269, WT strain	Reference National Center of Lyon
CNRIΔ*dltA*	CNRI strain deficient in *dltA* gene	This study
CNRIΔ*dltA*comp	CNRIΔ*dltA* complemented with pRB473-*dlt*	This study
CNRII	HA-MRSA, SCCmec type II, HT20020599	Reference National Center of Lyon
CNRIII	HA-MRSA, SCCmec type III, HT20020272	Reference National Center of Lyon
CNRIV	HA-MRSA, SCCmec type IV, HT20040100	Reference National Center of Lyon
CNRV	HA-MRSA, SCCmec type V, ST20050087	Reference National Center of Lyon
CNRVI	HA-MRSA, SCCmec type VI, HT20020274	Reference National Center of Lyon
CNRVII	HA-MRSA, SCCmec type VII, ST20141090	Reference National Center of Lyon
CNRVIII	HA-MRSA, SCCmec type VIII, ST20112957	Reference National Center of Lyon
CNRIX	HA-MRSA, SCCmec type IX, ST20112958	Reference National Center of Lyon
CNRX	HA-MRSA, SCCmec type X, ST20112959	Reference National Center of Lyon
RN4220	Deficient restriction strain used as intermediate cloning	31
Plasmids		
pMADΔ*dltA*	Plasmid pMAD carrying the truncated *dltA* gene	12
pRB473-*dlt*	pRB473 harboring dlt operon from *Staphylococcus xylosus*; CHL^R	16

CA-MRSA, community-acquired MRSA; HA-MRSA, hospital-associated MRSA.

necessary to a final concentration of 1 mM. Inoculum size was between 5×10^5 and 5×10^6 cfu/mL and checked by plate counting. The MICs and the clinical susceptibility were determined as defined by the CLSI standards.

For bacterial survival, cells were distributed to obtain around 2×10^8 cfu/mL per well and treated with antibiotic and DltA inhibitor (1 mM) when needed, as described in Coupri *et al.*[14]

Biofilm inhibition and eradication

The biofilm inhibition (BI) and biofilm eradication (BE) experiments were performed by microbroth dilution assays in 96-well polystyrene plates (Bio-Rad, CA, USA). Assays were done in BHI media supplemented to contain 2% glucose (BHI-G). Early-log phase cultures were adjusted to a final OD_{600} of 0.1 before inoculation. For BI assays, antibiotics and the DltA inhibitor were added when needed at the same time as inoculum. After 24 h at 37°C, biofilms were quantified by crystal violet (CV) staining. Briefly, the wells were gently washed twice with saline solution, air-dried for 45 min under laminar flow at room temperature and stained with 0.1% CV. CV was removed carefully, and biofilms were washed three times in saline solution, air-dried for 45 min before adding 30% acetic acid to extract the CV. Biofilm was resuspended vigorously after 15 min in contact with acetic acid and the optical density was measured at 570 nm. For BE assays, biofilms formed after 24 h of culture were washed and treated with antibiotics, inhibitor or medium. Microplates were incubated for 24 h at 37°C before determining the amount of biofilm by CV staining.

Infection model and antibiotic/adjuvant treatment

The animal model used in this study is based on the larvae of the insect *Galleria mellonella*. The experiments to determine virulence and effects of treatments were conducted as described previously.[14]

Quantification of D-alanine ester content in teichoic acids

Ester-linked D-alanine content was determined as described previously[17] with the following modifications: cultures were grown in 5 mL of BHI for 24 h at 37°C with shaking (120 rpm) and the DltA inhibitor was added when appropriate to a final concentration of 1 mM. Results represent the average of three independent experiments, each experiment tested duplicate samples.

Statistical analysis

Tests of statistical significance for MIC data were performed by two-tailed unpaired *t* test,[8] with GraphPad Prism 7.00. For the *Galleria* experiments, a log-rank test with a Bonferroni adjustment was performed for multiple comparison between all the different conditions used.[18]

Results

D-Alanylation deficiency of TAs marginally decreases vancomycin MICs of VISA strains

As shown in Table S1 (available as Supplementary data at *JAC* Online), some of the strains (CHU17, CNRI and CNRV) used in the present study are vancomycin intermediate-resistant *S. aureus* (VISA) with MICs of 4–8 mg/L. So, we wondered if D-alanylation deficiency treatment would decrease the MICs of vancomycin for these VISA strains. We initiated construction of Δ*dltA* mutants, which was successful only for the CNRI strain and the non-VISA strain MW2. Furthermore, the previously described DltA inhibitor[13] was synthesized. This inhibitor proved to be efficient in *S. aureus* strains since it decreased D-alanylation of teichoic acid by 70% to 100% (Table 2). However, genetic inactivation or chemical inhibition of D-alanylation only marginally decreased MICs of vancomycin for these VISA strains (Table S1).

D-Alanylation deficiency of TAs re-sensitizes MRSA to oxacillin and imipenem

Next, we determined the MICs of four β-lactams (amoxicillin, oxacillin, cefotaxime and imipenem) against 13 MRSA strains [12 hospital-acquired (HA)-MRSA and one community-acquired (CA)-MRSA; Tables 1 and 3]. As shown in Table 3, these strains have different levels of resistance to the tested β-lactams, ranging from low to high resistance. The median MICs of imipenem, oxacillin, amoxicillin, and cefotaxime against this panel of MRSA strains were 16 mg/L, 64 mg/L, 128 mg/L and 128 mg/L, respectively (Figure S1). Then, we tested the effect of D-alanylation deficiency on β-lactam resistance. Note that compared with the corresponding WT strains, no difference in growth in BHI was observed with the MW2Δ*dltA* mutant whereas the CNRIΔ*dltA* mutant showed a slightly longer lag phase than the parental strain (Figure S2). Treatment with the DltA inhibitor had no effect on growth of both WT strains, MW2 and CNRI (Figure S2). The MICs of oxacillin, amoxicillin, cefotaxime, and imipenem decreased in the MW2Δ*dltA* and CNRIΔ*dltA* mutants (Table 3). *In trans* complementation completely restored the MIC of imipenem for the MW2Δ*dltA* mutant whereas this effect was partial for the CNRIΔ*dltA* mutant, likely due to plasmid instability in this strain (Table 3). All strains were also treated with the DltA inhibitor. Except for the low-level-resistant strain CNRVIII, presence of the DltA inhibitor during the antibiotic treatment led to a significant reduction (≥4-fold) in the MIC of oxacillin across the other MRSA strains tested, independent of the degree of resistance and the SCCmec type (Tables 1 and 3). The median MIC of oxacillin was 8 mg/L against this panel of MRSA strains (Figure S1). Under the same conditions, a decrease in MICs of ≥4-fold was observed in only 38% and 31% of the strains for amoxicillin and cefotaxime with median MICs of 128 mg/L and 64 mg/L, respectively (Table 3; Figure S1). Inhibition of D-alanylation decreased the MIC of imipenem across all tested strains resistant to the antibiotic by 8-fold to 256-fold resulting in MIC values between 0.03 mg/L and 1 mg/L (Table 3). The median MIC of imipenem was 0.25 mg/L against the tested strains (Figure S1). The comparison with the results obtained with the Δ*dltA* mutants of strains CNRI and MW2 showed that pharmacological inhibition decreased the MICs of imipenem to the same extent as genetic inactivation (Table 3). Moreover, the DltA inhibitor had no additional effect on the Δ*dltA* mutants, suggesting the absence of any off-target effect of the molecule. We concluded that the DltA inhibitor acts as an anti-resistance molecule and its combination with imipenem restored β-lactam clinical susceptibility (MIC < 4 mg/L).

The DltA inhibitor potentiates the bactericidal effect of imipenem in S. aureus

Next, we evaluated the bactericidal impact of the antibiotics on WT and D-alanylation-deficient MRSA strains. Viable counts of WT strain MW2 and its isogenic Δ*dltA* mutant (MW2Δ*dltA*) were determined after 24 h in the absence or presence of imipenem (Figure 1), oxacillin, cefotaxime, or amoxicillin (Figure S3). In the absence of antibiotic, plate counts after 24 h were comparable for the WT and MW2Δ*dltA* mutant strains (Figure 1 and Figure S3).

Table 2. Percentage reduction of D-alanine ester content of teichoic acids in *S. aureus*

	Bacterial isolates						
	CNRI		MW2				
Characteristic	WT	Δ*dltA*	WT	Δ*dltA*	CNRIII	CNRVII	CNRIX
D-Alanine reduction, % (SD)[a]	86.7 (10.5)	101.5 (3.8)	70.7 (17.4)	101.5 (1.9)	87.2 (8.4)	100.4 (6.6)	92.2 (4.9)

[a]Ester-linked D-alanine was quantified from 24 h cultures of *S. aureus* WT strains cultivated in absence or presence of 1 mM DltA inhibitor or their isogenic mutants Δ*dltA*. The percentage reduction was determined as the ratio of D-alanine content after pharmacological or genetic inactivation of D-alanylation and that of WT strain without treatment. Results represent the average of three independent experiments, each experiment tested in duplicate samples.

Table 3. Inhibition of D-alanylation of teichoic acids reduces MIC (mg/L) of various β–lactams against MRSA-clinical isolates of *S. aureus*

	CTX		IPM		AMX		OXA	
Strain	INH−	INH+	INH−	INH+	INH−	INH+	INH−	INH+
CHU1	1024	8	32 (32[a])	0.25 (1[a])	64	4	256	2
CHU17	2048	1024	32	0.25	32	32	256	32
CNRI	2048	1024	16 (32[a])	0.25 (0.5[a])	32	32	256	64
CNRIΔ*dltA*	128	ND	0.25 (0.5[a]; 4[b])	0.25 (0.5[a])	16	ND	32	ND
CNRII	64	256	16	1	1024	128	32	8
CNRIII	512	128	64 (64[a])	1 (0.5[a])	1024	128	256	16
CNRIV	512	256	64 (32[a])	1 (1[a])	1024	128	128	16
CNRV	64	16	4	0.06	1024	128	4	1
CNRVI	32	16	0.5	0.03	64	64	4	1
CNRVII	64	64	16	0.25	128	128	32	8
CNRVIII	8	8	0.12	0.12	2	2	2	1
CNRIX	128	64	128	0.5	256	128	64	16
CNRX	2048	256	64	0.25	512	512	128	16
MW2	32	16	0.25 (0.5[a])	0.03 (0.03[a])	64	32	8	2
MW2Δ*dltA*	8	ND	0.03 (0.03[a]; 2[b])	0.03 (0.03[a])	8	ND	0.5	ND

MIC values are the average of three independent replicates. Abbreviations: CTX, cefotaxime; IPM, imipenem; AMX, amoxicillin; OXA, oxacillin; INH+, with DltA inhibitor; INH−, without DltA inhibitor; ND, not determined.
[a]MICs determined in MH medium.
[b]MICs of the Δ*dltA* complemented strains.

Survival of the WT strain treated with the selected antibiotics remained close to 100%. Survival of the MW2Δ*dltA* mutant was slightly reduced (<1 log) in presence of different amoxicillin concentrations (10 to 50 mg/L) (Figure S3A). In the case of oxacillin or cefotaxime, significant mortality of 2 to 5 log of the Δ*dltA* mutant was observed only with clinically irrelevant concentrations (≥30 mg/L) (Figure S3B and C). In contrast, imipenem was highly bactericidal for the MW2Δ*dltA* mutant. Compared with the WT strain, the survival after 24 h of treatment of the mutant dropped by around 3 log at 0.1 mg/L and 6 log at 0.5 mg/L and 1.0 mg/L of imipenem (Figure 1). Then we analysed the effect of pharmacological inhibition of D-alanylation in WT strains. In absence of imipenem, survival of all strains after 24 h in the presence of the DltA inhibitor was comparable (Fig. 1 and S3). Similar to the results obtained with the MW2Δ*dltA* mutant, chemical inhibition of D-alanylation by means of 1 mM of the DltA inhibitor strongly reduced the survival of the WT strain MW2 in the presence of imipenem by around 3 log at 0.1 mg/L and 5 log at 1 mg/L of the antibiotic (Figure 1). We concluded that in this strain the DltA inhibitor significantly potentiated the bactericidal effect of imipenem. However, killing by this combination was less efficient for the other three strains tested. Survival of CHU1 and CNRIII dropped by around 1 log and 1.5 log, respectively (Figure S4A and B), whereas the difference in killing of the CNRIV clinical isolate in the absence or presence of the inhibitor seems to be statistically not significant (Figure S4C).

Imipenem/DltA inhibitor combination decreases killing of insect larvae by MRSA

To evaluate the ability of inhibition of D-alanylation to overcome antibiotic resistance of MRSA in a host organism, we used the *G. mellonella* animal model. Larvae were infected with high doses (around 10^6 cfu/larva) of CA- (MW2) or HA-MRSA (CHU1, CNRI,

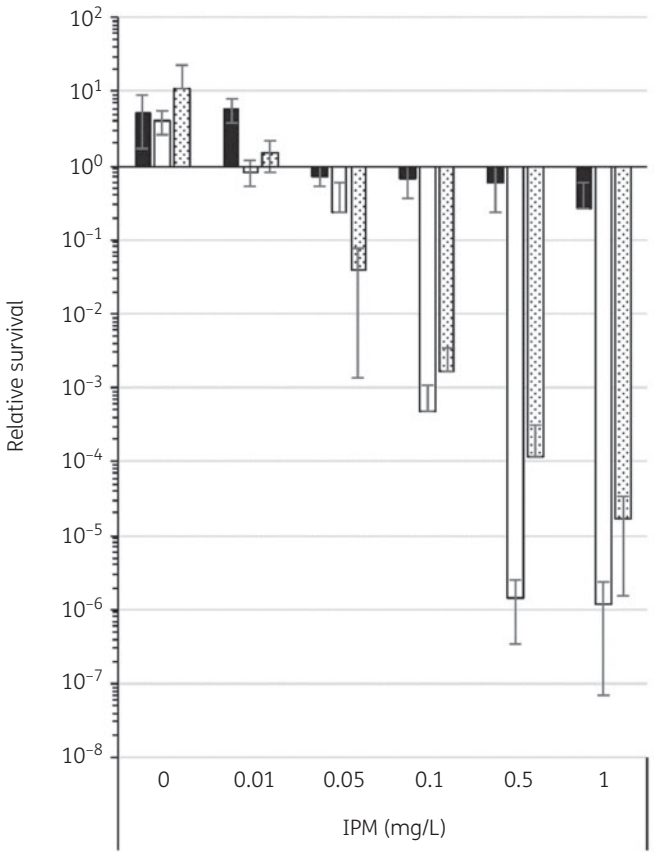

Figure 1. D-Alanylation deficiency of teichoic acids potentiates the bactericidal effect of imipenem in *S. aureus*. Early log-phase cultures of *S. aureus* MW2 (WT strain, black bars) in which D-alanylation was genetically inactivated (MW2Δ*dltA*, white bars) or pharmacologically inhibited (dotted bars) were treated with imipenem (IPM, from 0.01 to 1 mg/L) in the absence (unmarked bars) or presence (dotted bars) of DltA inhibitor (1 mM). Enumeration by plate counting was performed before addition of imipenem (T_0) and after 24 h of the different treatments at 37°C (T_{24}). The relative survival was determined as T_{24}/T_0. The results shown are the averages of three independent experiments.

CNRIII and CNRIV) WT strains or Δ*dltA* mutants (MW2Δ*dltA* or CNRI Δ*dltA*). Two hours post-infection, saline solution or antibiotic or DltA inhibitor or antibiotic/inhibitor combination was administered. The survival of the larvae was determined every 4 h from 12 to 48 h post-infection. Without treatment, the *S. aureus* CNR IV WT strain seems slightly less virulent than the other WT isolates but all strains killed the larvae and survival rates were between 0% and 17.5% after 48 h (Figure 2 and Figure S5). As shown in Figure 2, CNRIΔ*dltA* and MW2Δ*dltA* mutants were much less virulent in this insect model than their corresponding WT strains. 80% and 60% of the larvae were still alive after 48 h, respectively. It should be noted that decreased virulence of an *S. aureus* Δ*dltA* mutant was also reported in a *Drosophila* model.[19] Overall, administration of antibiotics alone did not cure larvae of infection and treatment with the DltA inhibitor alone only slightly increased the survival of the MW2 and CNRI-infected larvae 48 h post-infection, with survival rates of around 15% and 25%, respectively (Figure 2 and Figure S5 and Table S2). The treatment with the DltA inhibitor and cefotaxime improved the survival rate of CHU1-infected larvae around 6-fold at 48 h post-infection compared with the antibiotic alone and control conditions (P value $<5.6 \times 10^{-5}$, Table S2) whereas this combination was less efficient for CNRIII and CNRIV-infected larvae (Figure S5 and Table S2). Combination therapy with the DltA inhibitor and either oxacillin or imipenem considerably increased survival of the infected larvae with all MRSA strains tested (Figure 2, Figure S5 and Table S2). The combination imipenem/DltA inhibitor was particularly efficient with survival rates of larvae infected with MW2 or CNRI isolates of 55% or 45% after 48 h post-infection, respectively (Figure 2). This showed that inhibition of DltA in combination with some β-lactams re-sensitized MRSA strains to these antibiotics in the *G. mellonella* model.

Effect of imipenem and D-alanylation deficiency on biofilm formation and eradication

A main virulence factor of *S. aureus* is the ability to form biofilms on abiotic (including medical devices) and biotic surfaces (heart valves, bones). It is well known that antibiotics are less effective on bacteria in biofilms and new therapeutic strategies to overcome these limitations are urgently needed.[20] Since a Δ*dltA* mutant of *S. aureus* strain ATCC 35556 was strongly affected in adherence on polar and apolar surfaces,[21] we wondered if D-alanylation deficiency combined with imipenem treatment would be efficient to inhibit biofilm formation and/or to eradicate existing biofilms. The biofilm inhibition and eradication experiments were carried out in BHI-G. Under these conditions, biofilm formation of the WT and mutant strains was comparable (Figure S6). Low concentrations of imipenem increased biofilm formation of the MW2 WT strain but strongly inhibited its formation in the MW2Δ*dltA* mutant (Figure 3a). Interestingly, a comparable decrease in biofilm formation was observed in the WT strain in the presence of the DltA inhibitor. The 90% minimum biofilm inhibition concentration ($MBIC_{90}$) of imipenem for the MW2 WT strain was around 1 mg/L whereas it decreased to 0.125 mg/L and 0.0625 mg/L for the WT strain in the presence of the DltA inhibitor and the MW2Δ*dltA* mutant, respectively (Figure 3a and Table S3). This showed that pharmacological inhibition of D-alanylation efficiently prevented biofilm formation.

In the case of biofilm eradication, no measurable decrease of biofilm was observed with the MW2 WT strain even with the highest imipenem concentrations tested whereas low concentrations of the antibiotic destroyed the biofilm of the MW2Δ*dltA* mutant strain (Figure 3b). The 90% minimum biofilm eradication concentration ($MBEC_{90}$) of imipenem for the mutant strain was 0.25 mg/L (Figure 3b and Table S3). However, chemical inhibition of D-alanylation combined with imipenem had no effect on WT biofilm eradication (Table S3).

Discussion

As emphasized by the WHO report in 2017, [2] continuous efforts for the research and development of new antibiotics and treatment strategies against MRSA infections remains necessary. We explored D-alanylation as a potential new Achilles' heel of drug-resistant *S. aureus* by a comprehensive genetic and phenotypic analysis of 13 clinical MRSA strains with distinct genetic backgrounds. This analysis was necessary because of conflicting data

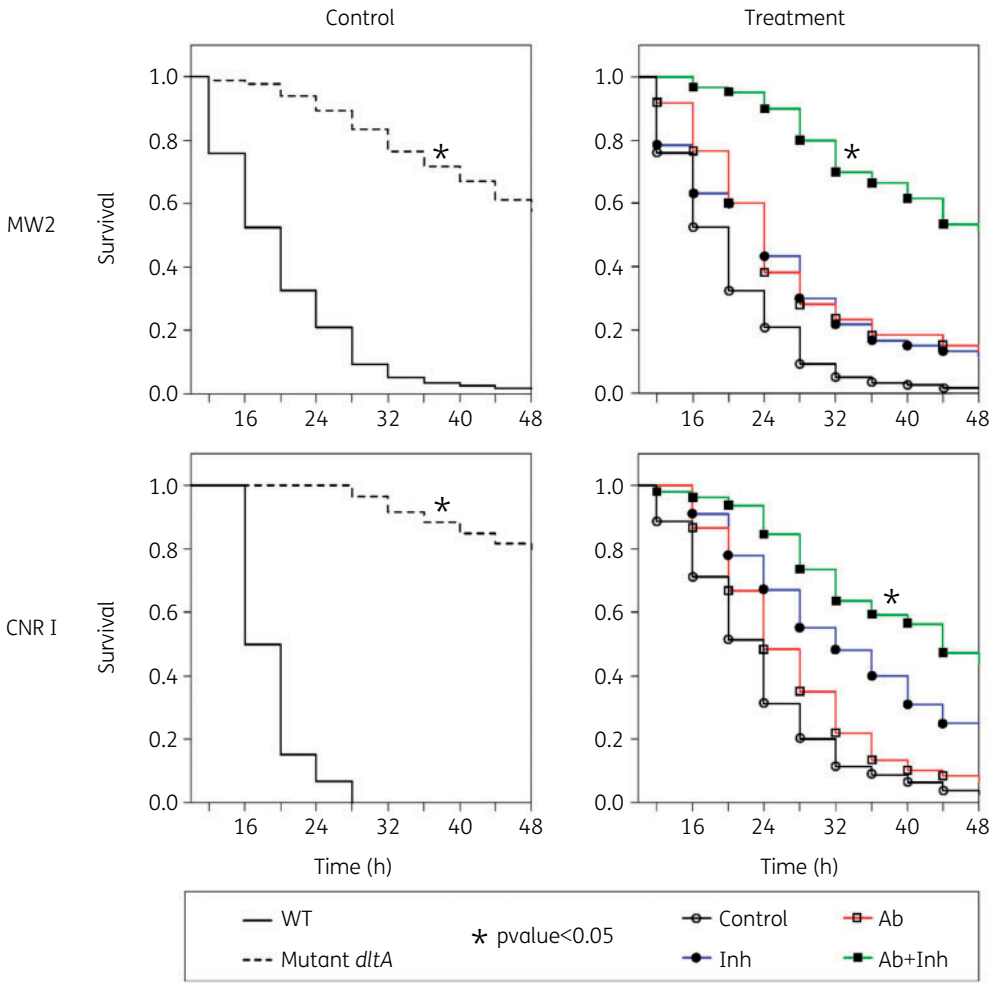

Figure 2. Imipenem/DltA inhibitor combination decreases killing of *Galleria mellonella* larvae by MRSA. *G. mellonella* larvae were infected with MRSA MW2 or CNRI WT strains (solid line) and their corresponding Δ*dltA* mutants (dotted line), followed 2 h post-infection by injections of 10 µL of saline solution (Control; black line) or imipenem at 0.6 mg/kg (Ab; red line) or DltA inhibitor at 48.5 mg/kg (Inh; blue line) or imipenem/inhibitor combination (Ab+Inh; green line). Living and dead larvae were counted every 4 h, from 12 to 48 h post-infection. At least 60 animals per condition were treated. Data are presented as Kaplan–Meier curves and analysis was performed with statistical software R (http://www.R-project.org/, Vienna, Austria). Curves with *P* values <0.05 were considered as statistically different and are indicated with an asterisk. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

in the literature. In fact, some reports showed that inactivation of the *dlt* operon increased resistance to β-lactams in *S. aureus*[22] whereas other studies considered the D-alanylation system of pharmacological relevance for infection control of MRSA.[12,13] Our results showed that the D-alanylation system is druggable using the DltA inhibitor, which has strong affinity for the DltA protein of *B. subtilis*,[13] since it significantly decreased D-alanylation of teichoic acids in *S. aureus*. This inhibitor displayed a synergy with β-lactams, especially with imipenem. Indeed, imipenem in combination with the DltA inhibitor decreased MICs to below the clinical breakpoint in all high-level resistant MRSA strains tested. Of note, the presence of the inhibitor did not decrease the MIC of imipenem against the strain CNRVIII, likely due to the already high intrinsic susceptibility to imipenem of this strain. Despite its high affinity for DltA *in vitro*, lead optimization is necessary since relatively high concentrations of the current inhibitor were needed to significantly potentiate β-lactam action.

We previously conducted comparable work in enterococci.[14] In contrast to the results shown here, genetic or chemical inhibition of D-alanylation did not modify the MICs of the enterococcal strains and only combinations with some antibiotics considerably increased their killing. This demonstrated that D-alanylation deficiency differently affects antibiotic resistance in different species.

A fundamental question is why does D-alanylation deficiency decrease β-lactam resistance of MRSA strains despite the presence of PBP2a? Previous work by other groups showed that presence of PBP2a is not sufficient for resistance to β-lactams of MRSA but also depends on several auxiliary genes, most of these having been shown to be implicated in peptidoglycan precursor synthesis and turnover.[23–26] Furthermore, it has been shown that PBP2a directly binds WTAs *in vitro*.[27] WTAs are important in regulating cell division in *S. aureus* and are also involved in maintaining resistance to β-lactams in MRSA strains.[28] Assuming that, in order to be active, PBP2a has to be recruited by WTAs and that binding to the

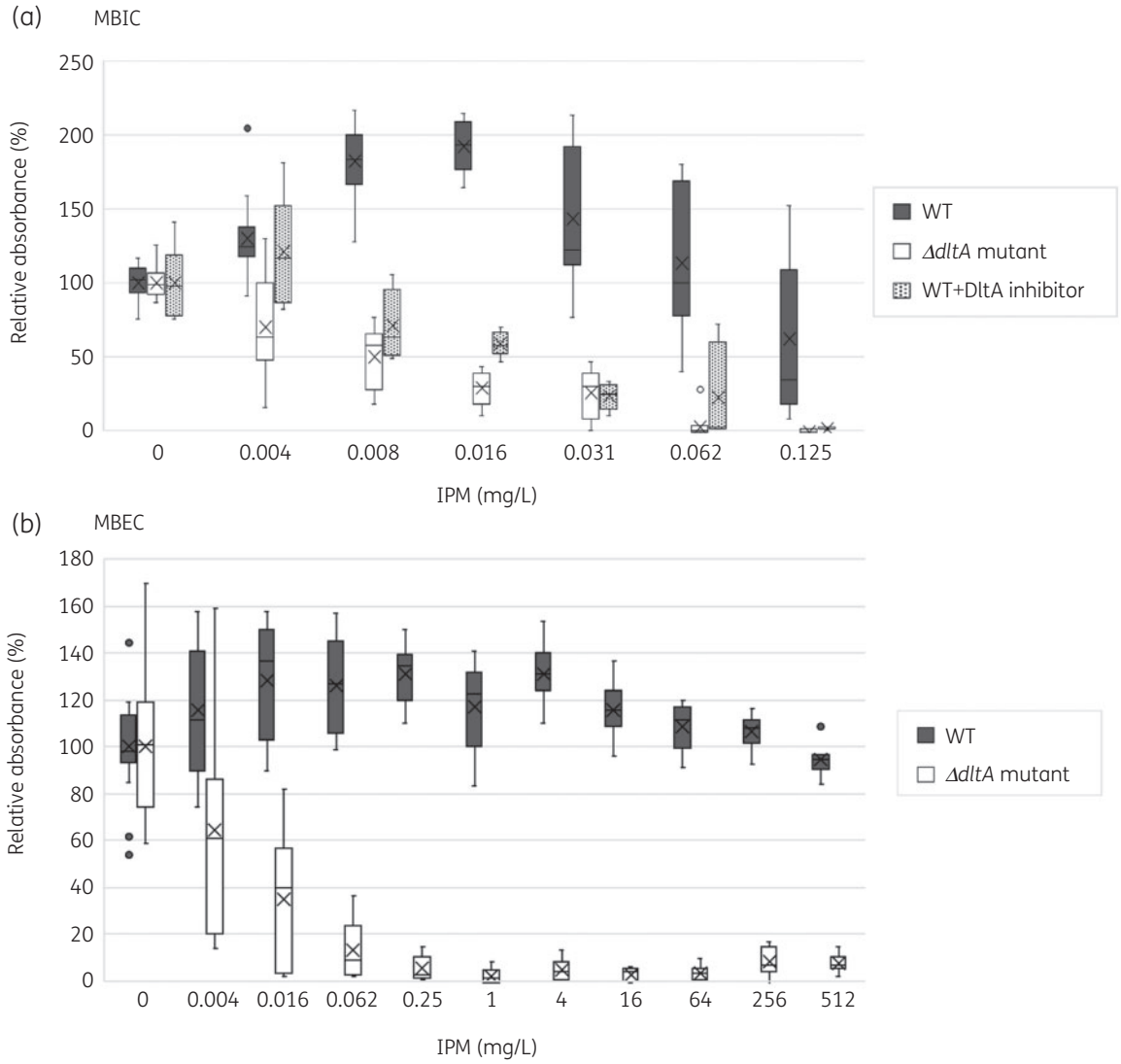

Figure 3. Anti-biofilm effect of DltA deficiency/imipenem combination in *S. aureus*. Minimum biofilm inhibitory concentration (MBIC) (a) and minimum biofilm eradication concentration (MBEC) (b). Concentrations of imipenem (IPM) against *S. aureus* MW2 (dark boxes), its Δ*dltA* mutant (white boxes), and the WT strain in presence of 1 mM of DltA inhibitor (dotted boxes) are shown. Assays were performed in BHI supplemented with 2% glucose (BHI-G). For the determination of $MBIC_{90}$ of the WT strain, a wider range of imipenem concentrations as shown in (b) were used. Values of $MBIC_{90}$ and $MBEC_{90}$ are summarized in Table S3. Eight technical replicates of negative controls (medium only) and four or three technical replicates of samples and positive controls (samples without antibiotic) were performed. Data from all the different samples were corrected by subtracting the mean of negative controls. Then, the OD_{570nm} of each sample was divided by the mean of positive controls to calculate the relative absorbance. Data of relative absorbance from three experiments (two experiments with inhibitor) were represented in box plots, providing the distribution, outliers and paired data relations.[32]

polymers needs D-alanine decoration, their absence or reduced number achieved by genetic or chemical inhibition of the Dlt-system might explain re-sensitization to β-lactams. However, in that case one would expect that overall the MICs of the tested strains should decrease to the level of MSSA strains. This is observed with imipenem but, with some exceptions, not for the two penicillins and the cephalosporin tested.

The most effective drug combined with the DltA inhibitor in our study was imipenem followed by oxacillin and by cefotaxime and amoxicillin, respectively. It has been shown that β-lactams have different affinities for the PBPs of *S. aureus in vitro*.[29] Imipenem has high affinity for three PBPs (PBP1, PBP2 and PBP3). Oxacillin also binds to these PBPs but with reduced affinity compared with imipenem. Cefotaxime efficiently targets only PBP2 and amoxicillin was not tested in the study. If this also applies *in vivo*, then it would mean that potentiation of β-lactams in D-alanylation-deficient MRSA strains is the more efficient the more transpeptidase activities of the native PBPs are blocked by the antibiotic. This notion is supported by results obtained with cefdinir, which has high affinity for PBP2 and PBP3 but not for PBP1.[29] In contrast to imipenem, this cephalosporin does not restore clinical susceptibility of strains MW2 and CNR1 in presence of the DltA inhibitor (Table S4).

In conclusion, DltA inhibition should be of promising therapeutic utility especially in combination with carbapenems for restoring the therapeutic efficacy of this important class of antibiotics against methicillin-resistant staphylococci. The current inhibitor used in this study is a useful scaffold in order to develop compounds with improved *in vivo* efficiencies to inhibit DltA. However, a critical point which may limit clinical utility is spontaneous development of resistance to these compounds. Once DltA inhibitors with improved *in vivo* activity are available, the frequency of resistance will need to be determined. An advantage of the Dlt system is that the four proteins, DltA to DltD, are essential for D-alanylation of teichoic acids. Therefore, the risk of resistance development can be counteracted by inhibitors targeting Dlt proteins other than DltA. Interestingly, an inhibitor of DltB of *S. aureus* has been recently identified in a synthetic lethal approach but this compound is unfortunately toxic to eukaryotic cells due to inhibition of eukaryotic topoisomerases.[11] Our results encourage greater efforts to screen or rationally design new highly efficient molecules targeting the Dlt system. These molecules are of the utmost pharmacological interest to overcome MRSA and likely other infections due to Gram-positive pathogens such as *Staphylococcus epidermidis*, enterococci and perhaps *Clostridioides difficile* as well.

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Transparency declarations

None to declare.

Supplementary data

Tables S1 to S4 and Figures S1 to S6 are available as Supplementary data at *JAC* Online.

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