Inhibition of \( \delta \)-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 \( \delta \)-alanylation of teichoic acids (TAs) mediated by the *dlt* operon gene products might be a druggable target to overcome \( \beta \)-lactam-resistance of MRSA.

**Methods:** MICs and bactericidal effects of several \( \beta \)-lactam antibiotics were monitored in a panel of clinical MRSA strains with genetic or chemically induced deficiency in \( \delta \)-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 \( \beta \)-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 \( \delta \)-alanylation potentiated the effect of imipenem on inhibition and/or eradication of biofilm.

**Conclusions:** Our combined results show that highly efficient inhibitors of \( \delta \)-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 \( \beta \)-lactams, except for some last-generation cephalosporins. However, resistance to these last drugs in clinical MRSA isolates from patients has already been reported.\(^3\) \( \beta \)-Lactams target penicillin binding proteins (PBPs) by inhibiting their transpeptidase activity, thereby blocking cell wall biosynthesis and leading finally to cell death.\(^4,5\) In most clinical isolates, resistance to \( \beta \)-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 \( \beta \)-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.\(^3,8\) For example, genetic or biochemical inactivation of the first step of wall teichoic acid (WTA) biosynthesis (TarO) renders MRSA highly susceptible to \( \beta \)-lactam antibiotics.\(^8\) WTAs and lipoteichoic acids (LTAs) are negatively charged cell wall polymers of Gram-positive bacteria, tagged with \( \delta \)-alanine esters, which confer a positive charge on these structures.\(^9\) \( \delta \)-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.\(^{10}\) However, the MICs of different β-lactam antibiotics, which are neutral or negatively charged molecules, were only marginally affected in a ω-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 ω-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 ω-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 ω-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 ω-alanylation deficiency, biofilm eradication. Finally, we show that inhibition of ω-alanylation increased survival of infected insect larvae of _Galleria mellonella_ treated with β-lactam antibiotics, arguing that inhibition of ω-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 (S’-O-[N-(ω-alanyl)-sulfamoyl]-adenosine) was synthesized as described by May et al.\(^{15}\) and solubilized at 10 mM in sterilized pure water.

### Mutagenesis and complementation of dltA gene

Construction of ΔdltA mutants in the parental strains MW2 and CNRI of _S. aureus_ was performed with plasmid pMADΔdltA (Table 1).\(^{12}\) After pasing in _S. aureus_ RN4220,\(^{15}\) the pMADΔdltA 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 ΔdltA 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\(_{600}\) 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

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

| Common name | Characteristics | Origin/reference |
|-------------|-----------------|------------------|
| MW2         | CA-MRSA, WT strain | This study |
| MW2ΔdltA    | MW2 strain deficient in dltA gene | This study |
| MW2ΔdltAcomp| MW2ΔdltA 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 |
| CNRIΔdltA   | CNRI strain deficient in dltA gene | This study |
| CNRIΔdltAcomp| CNRIΔdltA 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 |
| CNRV1       | 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 | Reference National Center of Lyon |
| Plasmids    |                 |                  |
| pMADΔdltA   | Plasmid pMAD carrying the truncated dltA gene | This study |
| pRB473-dlt  | pRB473 harboring dlt operon from Staphylococcus xylosus; CHL | Reference National Center of Lyon |

CA-MRSA, community-acquired MRSA; HA-MRSA, hospital-associated MRSA.
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 OD600 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 \( \alpha \)-alanine ester content in teichoic acids

Ester-linked \( \alpha \)-alanine content was determined as described previously17 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

\( \alpha \)-Alanylation deficiency of TAs re-sensitizes MRSA to oxacillin and imipenem

Next, we determined the MICs of four \( \beta \)-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 \( \beta \)-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 \( \alpha \)-alanylation deficiency on \( \beta \)-lactam resistance. Note that compared with the corresponding WT strains, no difference in growth in BHI was observed with the MW2\( \Delta \)dltA mutant whereas the CNRI\( \Delta \)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 MW2\( \Delta \)dltA and CNRI\( \Delta \)dltA mutants (Table 3). In trans complementation completely restored the MIC of imipenem for the MW2\( \Delta \)dltA mutant whereas this effect was partial for the CNRI\( \Delta \)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-resistance strain CNR8, the 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 (Table 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 \( \alpha \)-alanylation decreased the MIC of imipenem across all tested strains resistant to the antibiotics by 0.5- to 2.7-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 \( \Delta \)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 \( \Delta \)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 \( \beta \)-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 \( \Delta \)dltA-deficient MRSA strains. Viable counts of WT strain MW2 and its isogenic \( \Delta \)dltA mutant (MW2\( \Delta \)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 MW2\( \Delta \)dltA mutant strains (Figure 1 and Figure S3).
Survival of the WT strain treated with the selected antibiotics remained close to 100%. Survival of the MW2 ΔdltA 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 ΔdltA mutant was observed only with clinically irrelevant concentrations (≥30 mg/L) (Figure S3B and C). In contrast, imipenem was highly bactericidal for the MW2 ΔdltA 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 5 log at 1 mg/L of the antibiotic (Figure 1). We concluded that in this strain the ΔdltA 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/ΔdltA inhibitor combination decreases killing of insect larvae by MRSA**

To evaluate the ability of inhibition of α-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 10⁶ cfu/larva) of CA- (MW2) or HA-MRSA (CHU1, CNRI, CNRII, CNRIII, CNRIX, CNRI, CNRX, CNRV, CNRVII, CNRVIII, CNRVII, CNRX, MW2, MW2 ΔdltA). Survival of the WT strain treated with the selected antibiotics remained close to 100%. Survival of the MW2 ΔdltA 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 ΔdltA mutant was observed only with clinically irrelevant concentrations (≥30 mg/L) (Figure S3B and C). In contrast, imipenem was highly bactericidal for the MW2 ΔdltA 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 5 log at 1 mg/L of the antibiotic (Figure 1). We concluded that in this strain the ΔdltA 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).
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 (MW2\( \Delta \)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.

**Figure 1.** \( \nu \)-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 \( \nu \)-alanylation was genetically inactivated (MW2\( \Delta \)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.

**Effect of imipenem and \( \nu \)-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. Since a \( \Delta \)dltA mutant of \( S. \) aureus strain ATCC 35556 was strongly affected in adherence on polar and apolar surfaces, we wondered if \( \nu \)-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 MW2\( \Delta \)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 MW2\( \Delta \)dltA mutant, respectively (Figure 3a and Table S3). This showed that pharmacological inhibition of \( \nu \)-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 MW2\( \Delta \)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 \( \nu \)-alanylation combined with imipenem had no effect on WT biofilm eradication (Table S3).

**Discussion**

As emphasized by the WHO report in 2017, continuous efforts for the research and development of new antibiotics and treatment strategies against MRSA infections remains necessary. We explored \( \nu \)-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...
in the literature. In fact, some reports showed that inactivation of the dlt operon increased resistance to β-lactams in S. aureus whereas other studies considered the D-alanylation system of pharmacological relevance for infection control of MRSA. 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, 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. 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. Furthermore, it has been shown that PBP2a directly binds WTAs in vitro. WTAs are important in regulating cell division in S. aureus and are also involved in maintaining resistance to β-lactams in MRSA strains. Assuming that, in order to be active, PBP2a has to be recruited by WTAs and that binding to the

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 ΔdltA 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.
polymers needs α-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. 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 α-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. In contrast to imipenem, this cephalosporin does not restore clinical susceptibility of strains MW2 and CNR1 in presence of the DltA inhibitor (Table S4).

![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 ΔdltA 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 MBIC90 of the WT strain, a wider range of imipenem concentrations as shown in (b) were used. Values of MBIC90 and MBEC90 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 OD570nm 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.](https://academic.oup.com/jac/article/76/11/2778/6358701)
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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