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Intracellular forms of menadione-dependent small-colony variants of methicillin-resistant *Staphylococcus aureus* are hypersusceptible to β-lactams in a THP-1 cell model due to cooperation between vacuolar acidic pH and oxidant species

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Objectives: Phagocytosed methicillin-resistant *Staphylococcus aureus* (MRSA) are susceptible to β-lactams because of an acid-induced conformational change of penicillin-binding protein (PBP) 2a within phagolysosomes. We have examined whether this mechanism applies to *menD* and *hemB* small-colony variants (SCVs) of the COL MRSA strain, using cloxacillin, meropenem, doripenem, and vancomycin as comparator.

Methods: Intracellularly, the change in cfu from post-phagocytosis inoculum was measured after 24 h of incubation with antibiotics combined or not with *N*-acetylcysteine (NAC; oxidant species scavenger); the relative potency (C_s) was calculated from the Hill equation of concentration–response curves. Extracellularly, the effect of a pre-incubation with H_2O_2 was determined on MICs and killing at pH 7.4 and 5.5.

Results: Intracellularly, the β-lactam C_s was similar for the COL strain and the *hemB* mutant and not modified or slightly decreased (2- to 16-fold) by NAC. In contrast, the C_s was 100- to 900-fold lower for the *menD* mutant, but similar to that for the COL strain when NAC was present. Extracellularly, β-lactam MICs were markedly reduced at pH 5.5 for the parental strain and the haemin-supplemented *hemB* mutant, with limited additional effect of pre-incubation with H_2O_2. In contrast, MICs remained elevated at pH 5.5 for the *menD* mutant (supplemented with menadione sodium bisulphite or not), but were 7–10 dilutions lower after pre-incubation with H_2O_2. Vancomycin MICs were unaltered in all conditions, with no marked effect of NAC on C_s.

Conclusions: Cooperation between acidic pH and oxidant species confers high potency to β-lactams against intracellular forms of *menD* SCVs of MRSA.

Keywords: cloxacillin, meropenem, doripenem, H_2O_2, haemin

Introduction

Small-colony variants (SCVs) of *Staphylococcus aureus* are associated with persistent infections.[1] They have a particular tropism for the intracellular environment, which is often assumed to protect them from the action of most antibiotics.[1] In a recent study,[2] we examined the intracellular susceptibility of menadione- and haemin-dependent SCV stable mutants obtained from the methicillin-resistant *S. aureus* (MRSA) COL strain[3,4] to antistaphylococcal antibiotics. We observed that, although showing a much slower intracellular growth than the other strains, the *menD* mutant was as susceptible as its isogenic wild-type parent (no change in maximal relative efficacy and slight increase in relative potency, as determined by concentration–effect experiments). β-Lactams were not included in this study, because of the MRSA character of the strain. However, previous work in our laboratory has demonstrated that β-lactams in general, and cloxacillin and carbapenems in particular, regain full activity (both in terms of maximal relative efficacy and relative potency when tested against MRSA phagocytosed by THP-1 macrophages).[5,6] This surprising effect has been ascribed to the acidic pH prevailing in the phagolysosomes where the

bacteria sojourn and thrive, which causes penicillin-binding protein (PBP) 2a to undergo a conformational change from a closed to an open state that allows its acylation by β-lactams.[7]

We wondered whether a similar mechanism would also apply to SCVs. For this purpose, we examined and report here on the activity of three β-lactams (cloxacillin, meropenem and doripenem) against the intracellular forms of the *menD* and *hemB* mutants of the COL strain. Vancomycin was used as an anti-MRSA antibiotic acting also on cell wall synthesis, but through a distinct mechanism. While the activity of all three β-lactams was restored against the phagocytosed *hemB* mutant and its parental COL strain as anticipated, we made the unexpected observation that the same antibiotics became almost 100 to 900-fold more potent against the phagocytosed *menD* mutant than against its parental strain or the *hemB* mutant. A similar, although less impressive effect (10-fold increase) was also seen with a menadione-dependent SCV of the OM1a methicillin-susceptible *S. aureus* (MSSA) strain compared with its parental strain. We explain our observations as resulting from the combination of the direct effect exerted by acidic pH on the susceptibility of MRSA to β-lactams and of the hypersusceptibility of the *menD* mutant to the oxidative species produced by phagocytic cells.

Materials and methods

Antibiotics and main reagents

The following antibiotics were obtained as the branded products commercialized in Belgium for human use: gentamicin as Geomycin® and vancomycin as Vancocin® (both distributed in Belgium by GlaxoSmith-Kline, Wavre); meropenem as Meronem® (AstraZeneca, Brussels); and doripenem as Doribax® (Janssen-Cilag, Beerse, Belgium). Cloxacillin was purchased from Sigma–Aldrich (St Louis, MO, USA). Pooled human serum from healthy volunteers (used for opsonization) was purchased from Lonza Ltd (Basel, Switzerland) and stored at −80°C until use. Cell culture media and sera were from Invitrogen Corp. (Carlsbad, CA, USA), microbiological culture media from BD Bioscience (Franklin Lakes, NJ, USA) and other reagents from Sigma–Aldrich or Merck KGaA (Darmstadt, Germany).

Bacterial strains and susceptibility testing

We used two series of isogenic strains in this study, namely: (i) the *S. aureus* strain COL (wild-type hospital-acquired MRSA), its *menD* and *hemB* SCV mutants [constructed by allelic replacement with an *erm*(B) cassette-inactivated *hemB* gene and an *erm*(C) cassette-inactivated *menD* gene] and the *hemB* genetically complemented strain;[3,4] and (ii) a wild-type clinical MSSA isolate (OM1a) and its clonally related menadione-dependent SCV (OM1b), both recovered from a patient with chronic osteomyelitis.[8] MICs were determined following CLSI recommendations,[9] except that readings were made at 24 and 48 h for SCVs as previously described,[2] and incubations were made in Mueller–Hinton broth adjusted at either pH 7.4 or 5.5 (to mimic the pH of the extracellular medium and of the phagolysosomes, respectively).

Stability of β-lactams in broth at neutral and acidic pH

The chemical stability of cloxacillin and meropenem was tested after 24 h of incubation in Mueller–Hinton broth at pH 7.4 or adjusted at pH 5.5, using an HPLC assay [stationary phase: Lichrospher® 100 RP-18, 25×4 cm, 5 μM (Merck, Darmstadt, Germany); mobile phase: 25:75 (v/v) acetonitrile/25 mM phosphate buffer, pH 6.5; UV detection: 220 nm; linearity zone: 32–1000 mg/L (R^2=0.999) for both cloxacillin and meropenem].

Cells, cell infection and influence of N-acetylcysteine (NAC)

All experiments were conducted with human THP-1 cells [ATCC TIB-202 (ATCC, Manassas, VA, USA)], a myelomonocytic cell line displaying macrophage-like activity,[10] with culture conditions, cell infection and assessment of antibiotic activities performed exactly as described earlier.[2,11] Antibiotic activity was also examined in the presence of 25 mM NAC (Sigma–Aldrich), used as a scavenger of oxidant species produced by phagocytic cells.[12–14]

Determination of free concentrations of β-lactams in cell culture medium

β-Lactams were incubated for 2 h at 37°C in standard culture medium (RPMI-1640 plus 10% fetal bovine serum), after which free (unbound) antibiotic was separated by ultracentrifugation through a semi-permeable membrane with cut-off at 30 kDa (Amicon® Ultra-0.5 30K, EMD Millipore, Billerica, MA, USA). Free and total antibiotic concentrations were then measured by a disc-plate microbiological assay using Antibiotic Medium 11 at pH 7.95 and *S. aureus* strain ATCC 25923 as the test organism [zone of linearity of diameter versus $\log_{10}$ concentration: 4–512 mg/L for cloxacillin (R^2=0.963); 1–512 mg/L for meropenem (R^2=0.999); and 0.25–512 mg/L for doripenem (R^2=0.978)].

Influence of H_2O_2 on antibacterial activity

Bacteria were pre-exposed to 10 mM H_2O_2 in the dark (concentration selected to minimally affect bacterial viability),[15] after which the excess of H_2O_2 was destroyed by 100 mU/mL catalase. Bacteria were then pelleted, resuspended in fresh broth and either used directly for determination of MICs following CLSI recommendations or exposed to antibiotics for 5 h and plated for colony counting (assessment of bactericidal effect).

Curve-fitting and statistical analyses

Curve-fitting analyses were made using GraphPad Prism® version 4.03 (GraphPad Software, San Diego, CA, USA). Statistical analyses were made with GraphPad Instat version 3.06 (GraphPad Software). Hill equations were fitted to the data, allowing calculation of the following pertinent pharmacological descriptors, as described and discussed in our previous publications:[2,11] (i) the relative minimal efficacy [E_{min}; cfu increase in $\log_{10}$ units at 24 h compared with the original inoculum, as extrapolated for an infinitely low concentration of antibiotic (this parameter describes the actual bacterial growth in the absence of antibiotic)]; (ii) the relative maximal efficacy (E_{max}; cfu decrease in $\log_{10}$ units at 24 h compared with the original inoculum, as extrapolated for infinitely high antibiotic concentrations); and (iii) the drug static concentration (C_s, concentration of antibiotic resulting in no apparent bacterial growth compared with the original inoculum, as determined by graphical interpolation).

Results

Susceptibility testing

Table 1 shows the MICs of the three β-lactams and of vancomycin for the strains investigated, as determined in broth at pH 7.4 or 5.5 [to mimic the extracellular medium and the

Table 1. MICs of antibiotics and influence of medium supplementation with MSB or haemin (24/48 h)[a]

		MIC (mg/L)								
		MRSA COL						MSSA OM1		
			menD mutant		*hemB* mutant				OM1b	
Antibiotic	pH	wild-type	control	+MSB[b]	control	+haemin[b]	*hemB*gc	OM1a	control	+MSB[b]
Cloxacillin	7.4	128	128	128	128	128	128	0.25	0.125	0.25
	5.5	1/2	128	128	128	0.25	1/4	0.125	0.125	0.125
Doripenem	7.4	16/32	16/32	16/64	32/64	8/16	8/32	0.06	0.03	0.03
	5.5	0.5/2	8	8	8	0.25	0.5/1	0.03	0.03	0.03
Meropenem	7.4	32	32/64	32/64	32/64	16/32	16/32	0.125/0.25	0.06	0.125/0.25
	5.5	1/2	4/8	4/8	4/8	0.125/0.25	1	0.125	0.06	0.125
Vancomycin	7.4	1/2	1	1	1/2	1	1	1	0.5	0.5
	5.5	1/2	1	1	1	1	1	1	0.5/1	0.5

[a]Only values that were different at 48 h and at 24 h are indicated.
[b]2 mg/L.

phagolysosomal environment (infected intracellular compartment),[2] respectively]. For the COL strain and its *menD* and *hemB* mutants, β-lactam MICs were high at neutral pH (in accordance with their MRSA character). They were markedly reduced at acidic pH for the wild-type strain, its *hemB* mutant grown in haemin-supplemented broth and the genetically complemented *hemB* strain (*hemB*gc). In contrast, the MICs of cloxacillin were unchanged at pH 5.5 versus pH 7.4 for the *menD* and *hemB* mutants grown in non-supplemented medium and for the *menD* mutant cultured in medium supplemented with menadione bisulphite (MSB), while the MICs of the carbapenems were only partially reduced for these strains. For the OM1a MSSA strain, the MICs of all β-lactams were low and not different from those observed for the OM1b SCV, with no influence of pH or of supplementation with MSB. The vancomycin MICs were all low and ± 1 $\log_2$ dilution for all strains and in all conditions.

Influence of pH on β-lactam stability

To check that the huge difference in activity observed for some of the strains at neutral and acidic pH could not be attributed to chemical degradation at neutral pH, we compared the stability of cloxacillin and meropenem as an example carbapenem in Mueller–Hinton broth at pH 7.4 or 5.5 after 24 h of incubation at 37°C, using a drug concentration of 200 mg/L. Cloxacillin degradation reached only 8.8%±0.7% and 2.2%±1.4% at neutral and acidic pHs, respectively, and that of meropenem reached 23.9%±0.1% and 14.3%±0.0% in the same conditions. As the MICs and other activity parameters were determined on a $\log_2$-scale basis, these minor changes in drug concentration over time cannot account for the results observed.

Activity of antibiotics against intracellular S. aureus

Each strain was used to perform concentration-dependent experiments, with the change in cfu from the post-phagocytosis value determined after 24 h of incubation. For all strains, the data could be analysed and used to fit a Hill equation. Graphical representations are shown in Figure 1 (top panels) for the COL strain, the *menD* SCV [as such or with supplementation with MSB (*menD*s)], the *hemB* SCV and the corresponding complemented strain (*hemB*gc). Numerical values (regression parameters and pharmacological descriptors) are presented in Table S1 (available as Supplementary data at *JAC* Online). For β-lactams, no or only modest differences were seen between the COL strain, its *hemB* mutant and the *hemB*gc strain. Conversely and most strikingly, the *menD* mutant showed (i) a much lower intracellular growth (lower E_{min}) and (ii) a considerably increased potency (lower C_s) compared with its parental COL strain. Supplementation with menadione (*menD*s strain) reduced this effect, but the response was still significantly different from that of the COL strain. Vancomycin was also more potent against the MRSA *menD* strain compared with the parental strain, but the difference was less marked than that observed with β-lactams and was no longer seen after supplementation with menadione. Similar observations were made for the MSSA strain OM1a and its *menD* SCV mutant OM1b, although the difference in the relative potency was less important than with the MRSA strains and was entirely suppressed by the addition of menadione (OM1bs strain) (see Figure S1 and Table S2, both available as Supplementary data at *JAC* Online). Noteworthily, these differences in intracellular potencies were not directly correlated with commensurate differences in MICs (measured at pH 5.5). Thus, although β-lactams have the same MIC for the *menD* strain compared with the *hemB* and supplemented *menD* (*menD*s) strains, the latter two required higher β-lactam concentrations for controlling intracellular growth (see Figure S2, available as Supplementary data at *JAC* Online, where data of Figure 1 and Figure S1, available as Supplementary data at *JAC* Online are presented as a function of multiples of the MIC at pH 5.5). Most interestingly, the static concentration of β-lactams towards the MRSA *menD* mutant was 400 to 3300-fold lower than its MIC measured at acidic pH. This suggests that another factor specific to the intracellular environment contributed to the increased intracellular activity of β-lactams against this strain.

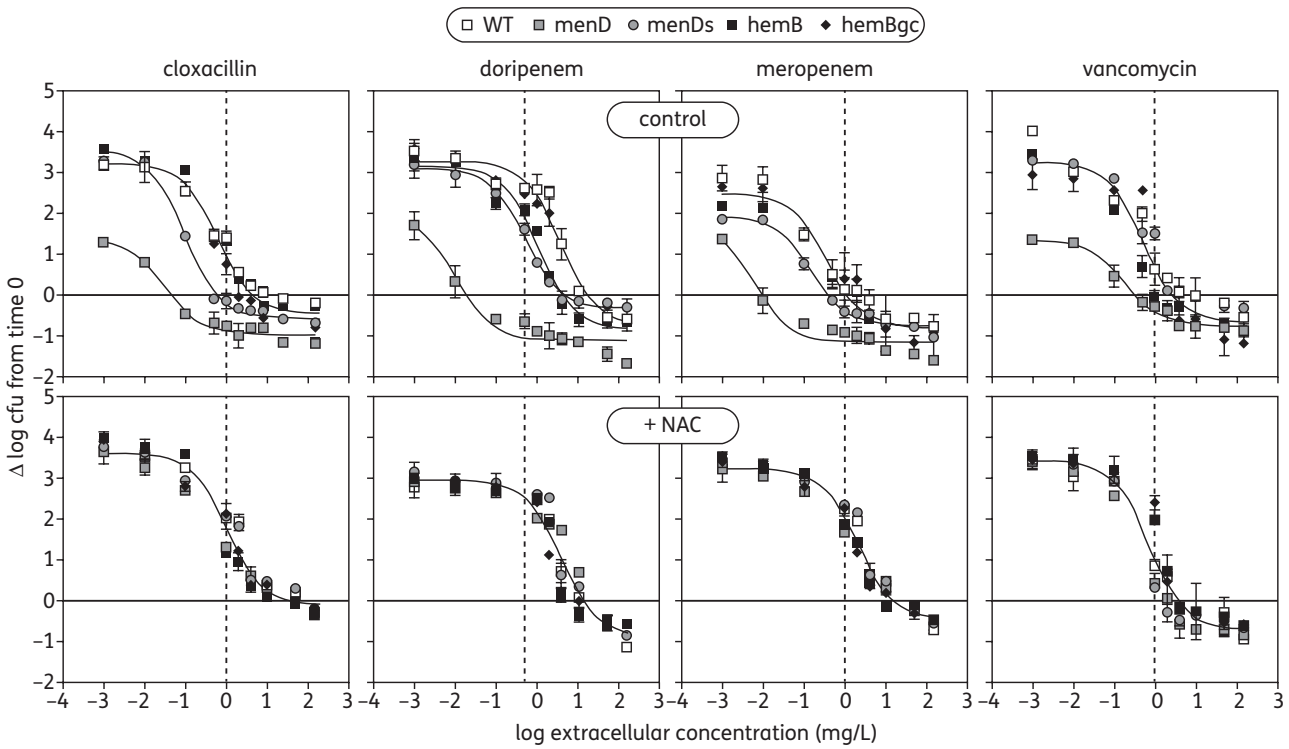

Figure 1. Concentration–response curves of cloxacillin, doripenem, meropenem and vancomycin against the intracellular forms of isogenic strains of *S. aureus* with an MRSA phenotype [wild-type (WT), *menD* mutant in control conditions (menD) or in medium supplemented with 2 mg/L MSB (menDs), *hemB* mutant (hemB) and the *hemB* genetically complemented mutant (hemBgc)]. Infected cells were incubated in the presence of increasing concentrations of antibiotics (total drug) for 24 h in control conditions (top panels) or in the presence of 25 mM NAC (bottom panels). The ordinate shows the change in the number of cfu (log scale) per mg of cell protein as compared with the initial inoculum. The continuous horizontal line corresponds to an apparent static effect. The abscissa shows the drug extracellular concentration. The broken vertical line corresponds to the MIC for the parental strain as measured at pH 5.5 (24 h reading). All values are means ± SD of three independent determinations (when not visible, the SD bars are smaller than the size of the symbols). Experiments have been reproduced three times with similar results. For clarity, individual curves are shown only for strains with data significantly differing ($P < 0.05$) from one another by repeated measures ANOVA followed by Tukey's multiple comparison test. When not statistically different, data from different strains were analysed together to calculate a single Hill function. Individual regression parameters and pharmacological descriptors are shown in Table S1, available as Supplementary data at *JAC* Online.

Determination of the free concentration of β-lactams

Because protein binding in human serum is known to be high and concentration-dependent for cloxacillin,[16] but minimal for carbapenems,[17] we measured the free fraction for these antibiotics under the conditions of our experiments (RPMI-1640 medium supplemented with 10% fetal bovine serum) at three concentrations (total drug) in the range of the human C_{max} (total drug). The carbapenem (meropenem and doripenem) free fraction was >80% at all three concentrations, and that of cloxacillin was 47%, 60% and 78% at 8, 16 and 32 mg/L (see Figure S3, available as Supplementary data at *JAC* Online). These differences are probably negligible compared with the magnitude of changes of activity observed.

Influence of NAC on antibiotic activity against intracellular S. aureus

In a concurrent investigation, we have shown that the oxidative burst produced by phagocytic cells could play an important role in the bactericidal effect of antibiotics towards SCVs.[18] We therefore examined the influence of NAC, a scavenger of oxidant species,[12–14] on the intracellular activity of β-lactams and of vancomycin against the *menD* strain in comparison with the other SCV strains and their parental wild-type bacteria. In preliminary experiments, we checked that NAC did not modify the MIC of antibiotics (no change noted; data not shown). We then performed concentration– response experiments for each strain in the presence of NAC. The data are shown in Figure 1 (bottom panels) for the MRSA COL strain and the corresponding SCVs, and in Figure S1, available as Supplementary data at *JAC* Online for the MSSA OM1a strain, with the corresponding numerical values of the pharmacological descriptors presented in Tables S1 and S2, available as Supplementary data at *JAC* Online. All strains behaved alike in the presence of NAC for each antibiotic tested and, specifically, with no statistically significant difference between the *menD* SCV and the OM1b SCV versus their corresponding COL and OM1a parental strains. Thus, not only the higher potency (lower C_s) of β-lactams (and vancomycin to a lower extent) towards the *menD* SCVs was no longer observed, but the intracellular growth of these strains (E_{min}) was brought to the same level as that of the parental

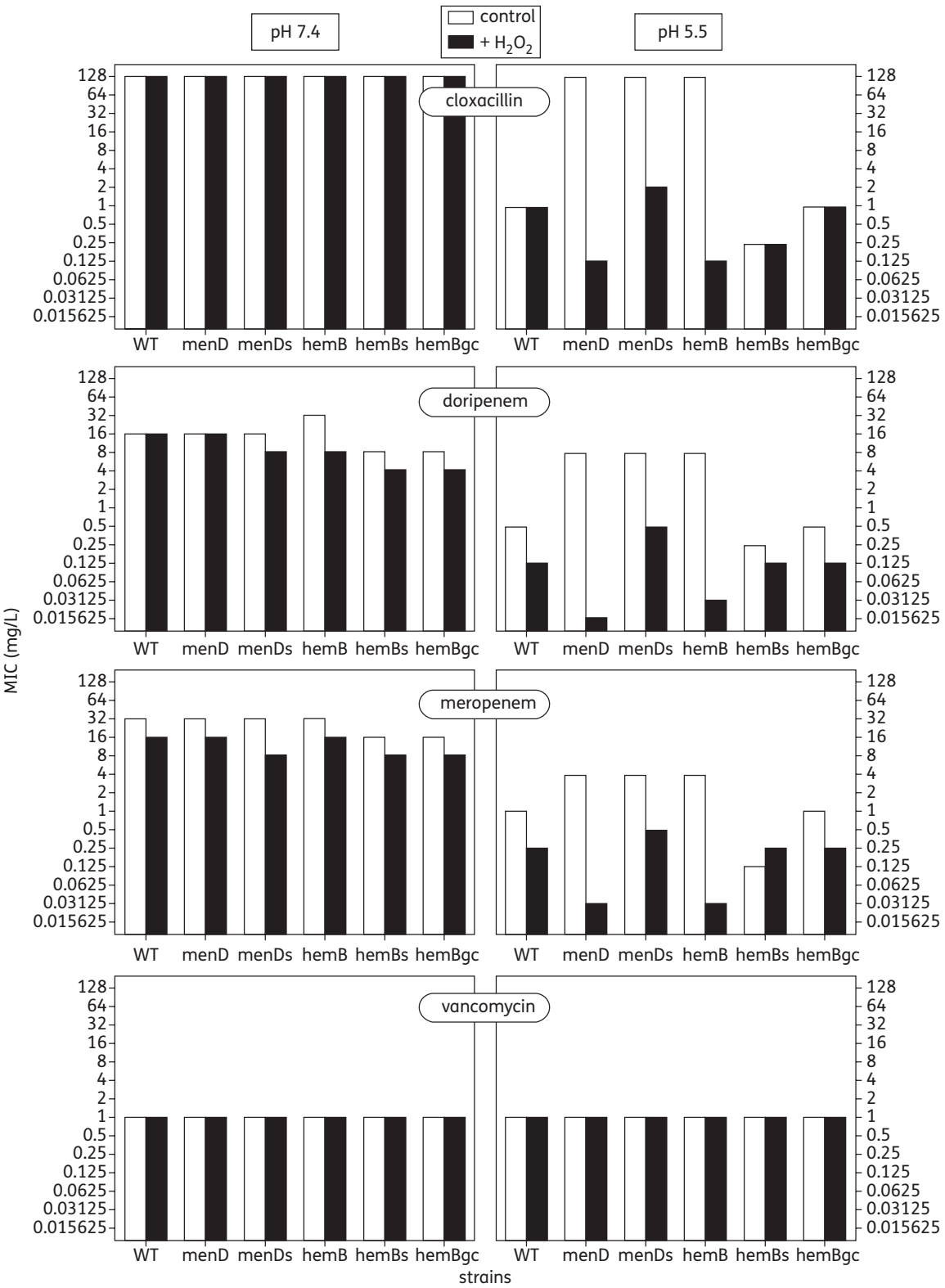

Figure 2. Influence of pH and H_2O_2 on the MICs of cloxacillin, doripenem, meropenem and vancomycin for the isogenic strains of *S. aureus* with an MRSA phenotype [wild-type (WT), *menD* mutant in control conditions (menD) or in medium supplemented with 2 mg/L MSB (menDs), *hemB* mutant in control conditions (hemB) or in medium supplemented with 2 mg/L haemin (hemBs) and the *hemB* genetically complemented mutant (hemBgc)]. MICs were read after 24 h of incubation in broth at pH 7.4 (left-hand panels) or 5.5 (right-hand panels) for bacteria that were pre-incubated (black bars) or not (white bars) with 10 mM H_2O_2 for 30 min prior to antibiotic addition. Experiments have been reproduced three times with similar results.

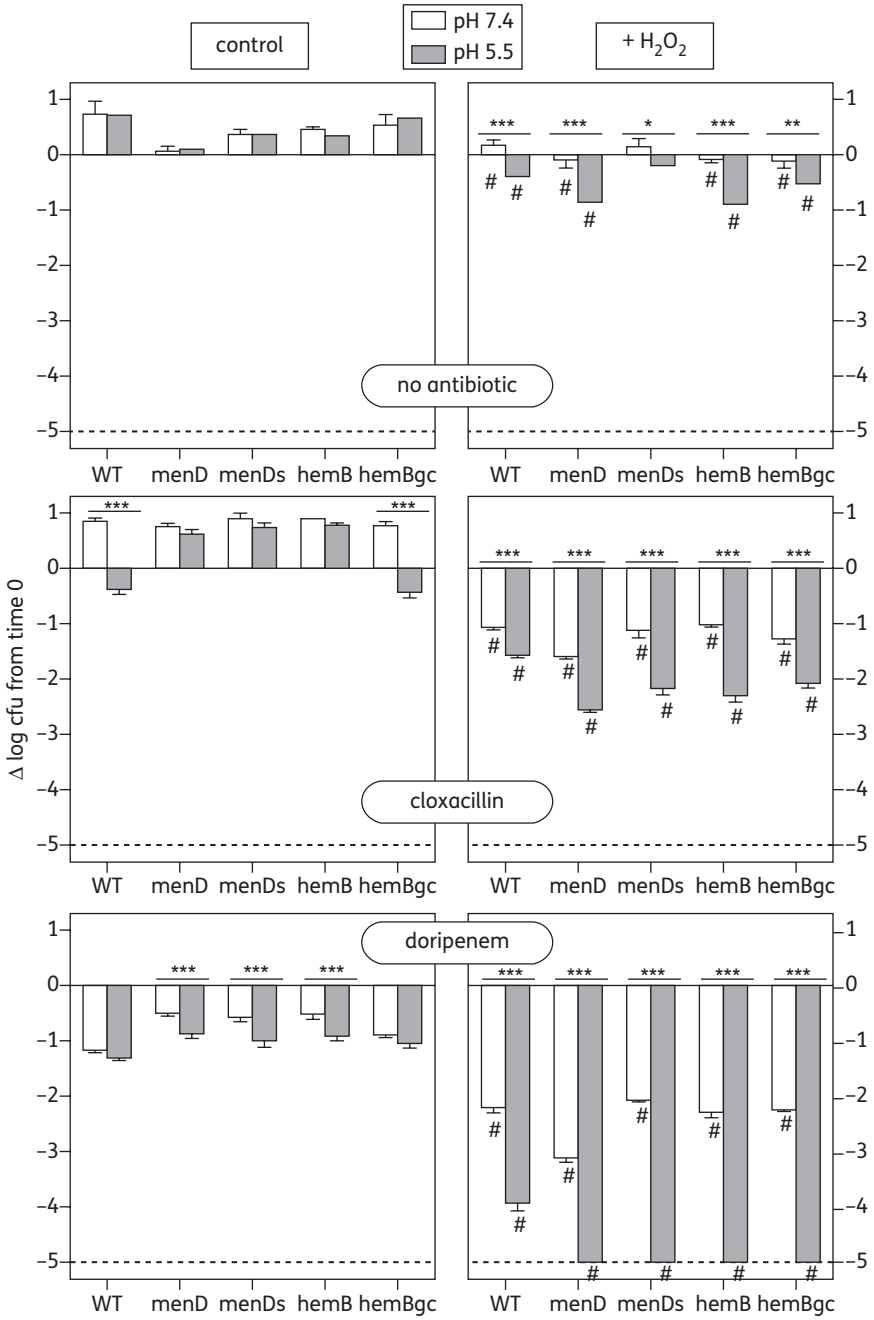

Figure 3. Influence of pH and H_2O_2 on the activity of antibiotics against the isogenic strains of *S. aureus* with an MRSA phenotype [wild-type (WT), *menD* mutant in control conditions (menD) or in medium supplemented with 2 mg/L MSB (menDs), *hemB* mutant (hemB) and the *hemB* genetically complemented mutant (hemBgc)]. Bacteria were pre-exposed (right-hand panels) or not (left-hand panels) to 10 mM H_2O_2 for 30 min, then incubated in broth at pH 7.4 (white bars) or 5.5 (grey bars) in the presence of antibiotics for 5 h at concentrations mimicking their human C_{max} (cloxacillin, 8 mg/L; doripenem, 23 mg/L; meropenem, 50 mg/L; and vancomycin, 50 mg/L). The ordinate shows the change in bacterial counts from the initial inoculum. The continuous horizontal line corresponds to an apparent static effect and the broken horizontal line corresponds to the limit of detection. All values are means ± SD of three independent determinations (when not visible, the SD bars are smaller than the size of the symbols). Experiments have been reproduced two times with similar results. Statistical analysis (ANOVA with Tukey's *post hoc* test): comparisons between pH 7.4 and pH 5.5 for each individual strain (*$P<0.05$, **$P<0.01$ and ***$P<0.001$); and comparisons between control conditions and pre-incubation with H_2O_2 for each individual strain at a given pH (#$P<0.05$).

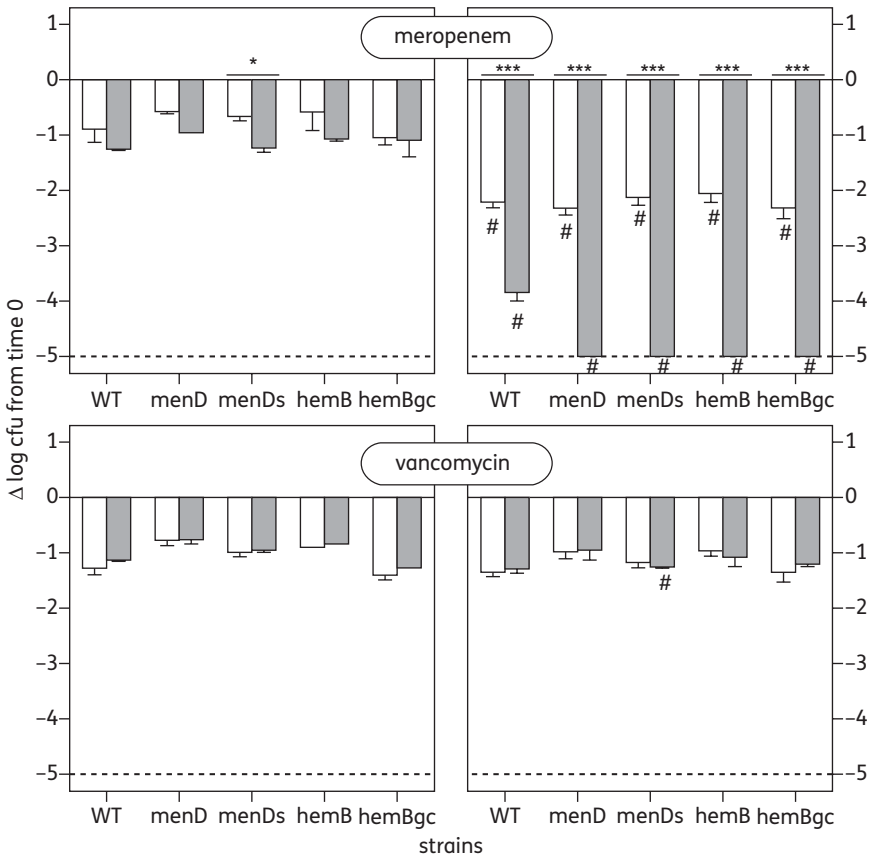

Figure 3. (Continued).

strains and of the other mutants. With the other strains, NAC also slightly reduced the relative potency of cloxacillin and meropenem, but barely affected that of vancomycin and of doripenem.

Influence of H_2O_2 on antibiotic activity against extracellular MRSA

As the previous experiment suggested a key role of oxidant species produced by THP-1 cells in the higher susceptibility of the *menD* MRSA strain towards β-lactams, we examined the effect of a 30 min pre-incubation of all MRSA strains in the presence of 10 mM H_2O_2 on the MICs of these antibiotics. Figure 2 shows that this pre-incubation did not modify the MICs of β-lactams when measured at neutral pH. At acidic pH, no or only a slight effect was seen for the wild-type strain. In contrast, major changes (7–10 dilutions) were seen for the *menD* and *hemB* mutants when tested at pH 5.5. Most conspicuously, the MICs of β-lactams for both SCVs were lower (2–3 $\log_2$ dilutions) than those for the wild-type strain measured at acidic pH. This decrease in MIC was less important for the *menD* strain grown in medium supplemented with menadione (*menD*s strain) and was not observed any longer for the *hemB* mutant when gown in medium supplemented with haemin (*hemB*s strain) or when genetically complemented (*hemB*gc strain). No effect of H_2O_2 on MICs was seen for vancomycin at either pH 7.4 or 5.5.

To further document the effect of H_2O_2 on bacterial killing, we also evaluated its influence at neutral and acidic pH on bacterial counts. To this effect, bacteria were exposed to H_2O_2 for 30 min and thereafter to antibiotics for 5 h [in the absence of H_2O_2; each antibiotic was used at a concentration mimicking its human C_{max} (note that the C_{max} value of cloxacillin was lower than its MIC at neutral pH)]. The results are shown in Figure 3. In the absence of H_2O_2, cloxacillin was ineffective and killing was limited to ~1–1.5 $\log_{10}$ cfu for doripenem, meropenem and vancomycin. Exposure to H_2O_2 only resulted in a very modest killing (≤1 $\log_{10}$ cfu) compared with controls, which was slightly more important for the *menD* and *hemB* mutants at acidic pH. In contrast, the combination of pre-exposure to H_2O_2 and incubation with antibiotics resulted in an enhanced killing for all three β-lactams at pH 7.4, and an apparent eradication for doripenem and meropenem with the SCV mutants at pH 5.5. No effect of H_2O_2 was seen for vancomycin. Testing the antibiotics at a concentration corresponding to their MICs showed similar differences in killing between H_2O_2-exposed and control bacteria (Figure S4, available as Supplementary data at *JAC* Online).

Discussion

SCVs are usually considered as poorly susceptible to antibiotics and refractory to their action when intracellular. Quite unexpectedly, the present data show that the intracellular environment

actually makes a menadione-dependent (*menD*) MRSA SCV hypersusceptible to β-lactams. Noticeably, this strain shows the same intracellular susceptibility as MSSA OM1b SCV (see C_s values in Tables S1 and S2, available as Supplementary data at *JAC* Online), and both are much more susceptible in cells than their corresponding parental strains. Thus, two mechanisms must be envisaged, namely a restoration of susceptibility to β-lactams for the MRSA *menD* SCV and another additional effect that benefits both MRSA and MSSA SCVs.

We showed previously that the activity of β-lactams can be restored against normal phenotype MRSA strains when these are phagocytosed by THP-1 cells, due to the acidic pH that prevails in phagolysosomes.[5,6] This pH effect has been reproduced in acellular systems and shown to be related to a pH-induced conformational change of PBP 2a.[7] This was not the case for the SCVs tested in this study, since acidifying the pH of the broth did not reduce their MICs. We have no simple explanation for this lack of restoration in broth, but suggest that it is linked to the SCV character of the strains, since a marked decrease in MICs could be obtained at acidic pH for the *hemB* SCV after haemin supplementation or complementation. For the *menD* SCV, MSB supplementation was not sufficient to restore the susceptibility to β-lactams at acidic pH, although it allowed for restoration of growth. This may be due to the fact that MSB supplementation does not fully reverse the SCV phenotype of this strain.[2] It shows, nevertheless, that growth impairment *per se* is not the main reason for lack of restoration of susceptibility. A most interesting aspect in our work is actually that restoration of the susceptibility of *menD* and *hemB* SCVs to β-lactams could be obtained by the combined effects of the addition of H_2O_2 and a decrease of pH. This identifies oxidative stress as a potential additional mechanism that could play a critical role intracellularly beyond the effect of pH.

After phagocytosis, *S. aureus* is exposed to the release of reactive oxygen species that are part of the natural defence of phagocytes against invading bacteria.[19–21] Oxidant stress reduces the NADH pool inside bacteria, with, as a consequence, a reduction in the ATP content and growth rate.[22,23] NADH serves as a cofactor for the reduction of menadione in menaquinone, which itself transfers electrons to the haem of cytochromes, providing the energy required for ATP release from the F_0F_1-ATPase.[22] The enzymes responsible for the synthesis of menaquinone and haem, respectively, are defective in *menD* and *hemB* mutants. This may explain why these strains can become incapable of coping with oxidant stress. SCVs, indeed, are known to be highly susceptible to oxidant agents, such as tellurite or selenite.[24] β-Lactams induce the production of oxidant species in bacteria,[25,26] which makes the following sequence of events quite likely: (i) after phagocytosis, *menD* SCV quickly becomes exposed to an oxidative stress, which it can only partially resist (hence its slower growth rate); (ii) β-lactams, because of the combined effect of oxygen reactive species (including H_2O_2) and of the low pH (as demonstrated in broth), will then exert an intense bactericidal effect in spite of the MRSA character of this strain. The increased potency of β-lactams towards the *menD* SCV is no longer observed if NAC is added to the incubation medium, which further supports the role of oxidant species. In this context, a clear correlation has indeed been demonstrated between the resistance of *S. aureus* to oxidative stress and to β-lactams,[27] and, conversely, between its susceptibility to H_2O_2 and to oxacillin.[28] The *hemB* SCV strain will not demonstrate a similar increase in intracellular susceptibility, because the intracellular milieu provides sufficient amounts of haem-like compounds to cause a local reversal of its phenotype. We show, indeed, that the intracellular behaviour of the *hemB* strain is similar to that of the parent strain, with no further modification by the addition of haemin or by complementation.[2] Vancomycin is also reported to trigger the production of oxidant species in bacteria, but only at high concentrations.[26] This is consistent with the lack of effect of NAC on vancomycin intracellular activity in our conditions.

As a conclusion, this paper documents for the first time that the menadione-dependent SCV mutant of an MRSA *S. aureus* strain is much more susceptible to β-lactams in the intracellular environment than are MRSA and MSSA strains with a normal phenotype, because of the combination of local acidic pH and exposure to oxidative burst. While the molecular mechanism(s) of this cooperation remain(s) to be determined, our observations may open research perspectives for the understanding of the metabolic defects occurring in these strains and, possibly, for the management of the infections they cause.

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Transparency declarations

None to declare.

Supplementary data

Tables S1 and S2 and Figures S1 to S4 are available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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Supplementary data

Table S1. Pertinent regression parameters[a] (with CIs) and statistical analysis[b] of the dose-response curves illustrated in Figure 1

	Control conditions						+NAC					
				C_s[e]						C_s[e]		
Antibiotic	strain	E_{min}[c]	E_{max}[d]	mg/L	× MIC (pH 5.5)	R^2	strain	E_{min}[c]	E_{max}[d]	mg/L	× MIC (pH 5.5)	R^2
Cloxacillin	wild-type	3.14 (2.89 to 3.39) a,A	-0.17 (-0.40 to 0.06) a,A	11.2	11.2	0.989	wild-type	3.57 (3.14 to 4.00) a,B	-0.21 (-0.70 to 0.29) a,A	27.3	27.3	0.974
	menD	1.35 (0.96 to 1.74) b,A	-0.96 (-1.11 to -0.81) b,A	0.04	0.0003	0.973	*menD*	3.37 (3.04 to 3.70) a,B	-0.07 (-0.38 to 0.25) a,B	34.5	0.27	0.985
	*menD*s	3.53 (3.32 to 3.74) a,A	-0.56 (-0.71 to -0.42) b,A	0.59	0.005	0.995	*menD*s	3.55 (3.08 to 4.02) a,A	-0.07 (-0.59 to 0.45) a,B	72.4	0.57	0.969
	hemB	3.51 (3.24 to 3.77) a,A	-0.39 (-0.63 to -0.15) a,A	5.50	22.0	0.991	*hemB*	3.98 (3.71 to 4.25) a,B	-0.20 (-0.45 to 0.05) a,A	12.3	49.2	0.936
	*hemB*gc	3.17 (3.01 to 3.34) a,A	-0.77 (-0.92 to -0.63) b,A	2.28	2.28	0.997	*hemB*gc	3.65 (3.17 to 4.12) a,B	-0.20 (-0.70 to 0.31) a,B	22.1	22.1	0.972
Doripenem	wild-type	3.27 (2.85 to 3.69) a,A	-0.84 (-1.48 to -0.20) a,A	17.8	71.2	0.967	wild-type	2.87 (2.51 to 3.23) a,A	-1.17 (-1.71 to -0.64) a,A	12.3	12.3	0.980
	menD	1.93 (0.91 to 2.95) b,A	-1.10 (-1.38 to -0.82) a,A	0.02	0.0025	0.919	*menD*	2.75 (2.48 to 3.02) a,B	-0.83 (-1.29 to -0.366) a,A	25.1	49.2	0.983
	*menD*s,	3.14 (2.87 to 3.41) a,A	-0.56 (-0.81 to -0.30) a,A	4.44	0.56	0.968	*menD*s,	3.13 (2.59 to 3.67) a,A	-0.96 (-1.77 to -0.16) a,A	15.5	3.14	0.956

	hemB	3.17 (2.71 to 3.63) a,A	-0.83 (-1.30 to -0.36) a,A	4.15	16.6	0.972	*hemB*	2.98 (2.34 to 3.63) a,A	-0.86 (-1.70 to -0.01) a,A	10.5	1.94	0.939
	*hemB*gc	3.31 (2.90 to 3.73) a,A	-0.59 (-1.16 to -0.03) a,A	16.22	32.4	0.968	*hemB*gc	3.05 (2.64 to 3.46) a,A	-0.74 (-1.24 to -0.24) a,A	9.33	42.0	0.976
Meropenem	wild-type	2.90 (2.50 to 3.31) a,A	-0.46 (-0.73 to -0.20) a,A	1.16	1.16	0.978	wild-type	3.19 (2.79 to 3.58) a,A	-0.59 (-1.11 to -0.08) a,A	15.7	18.7	0.975
	menD	1.75 (0.63 to 2.88) b,A	-1.14 (-1.39 to -0.89) b,A	0.01	0.0013	0.913	*menD*	3.06 (2.80 to 3.32) a,B	-0.37 (-0.68 to -0.07) a,B	14.5	1.81	0.988
	*menD*s	2.04 (1.76 to 2.32) b,A	-0.73 (-0.90 to -0.56) a,A	0.40	0.05	0.984	*menD*s	3.31 (2.87 to 3.74) a,B	-0.50 (-1.07 to 0.7) a,A	19.1	2.39	0.971
	hemB	2.16 (2.04 to 2.34) b,A	-0.84 (-0.96 to -0.72) a,A	1.03	4.12	0.996	*hemB*	3.40 (3.13 to 3.67) a,B	-0.42 (-0.73 to -0.12) a,B	12.2	48.8	0.990
	*hemB*gc	2.39 (1.78 to 3.01) a,A	-1.17 (-1.75 to -0.60) b,A	1.58	1.58	0.942	*hemB*gc	3.33 (2.96 to 3.71) a,B	-0.47 (-0.90 to -0.04) a,B	11.7	11.7	0.981
Vancomycin	wild-type	3.45 (2.80 to 4.09) a,A	-0.31 (-0.85 to 0.22) a,A	5.25	5.25	0.949	wild-type	3.29 (2.90 to 3.67) a,A	-0.64 (-1.01 to -0.27) a,A	3.80	3.80	0.984
	menD	1.36 (1.14 to 1.58) b,A	-0.74 (-0.88 to -0.60) a,A	0.34	0.34	0.984	*menD*	3.36 (3.16 to 3.55) a,B	-0.83 (-0.99 to -0.67) a,A	1.78	1.78	0.997
	*menD*s	3.32 (2.94 to 3.71) a,A	-0.35 (-0.69 to -0.00) a,A	6.02	6.02	0.979	*menD*s	3.61 (3.24 to 3.98) a,A	-0.67 (-0.96 to -0.38) a,A	1.82	1.82	0.990
	hemB	3.42 (3.11 to 3.57) a,A	-0.69 (-0.84 to -0.53) a,A	1.14	1.14	0.995	*hemB*	3.63 (3.25 to 4.00) a,A	-0.69 (-1.09 to -0.29) a,A	6.02	6.02	0.986
	*hemB*gc	3.06 (2.16 to 3.96) a,A	-1.19 (-2.04 to -0.35) b,A	1.20	1.20	0.915	*hemB*gc	3.47 (2.85 to 4.09) a,B	-0.79 (-1.46 to -0.12) a,A	5.82	5.82	0.961

[a]Calculated based on sigmoidal regressions with an Hill coefficient of 1 for extracellular data and for intracellular data with cloxacillin, doripenem, meropenem and vancomycin.

[b]Statistical analyses: (one-way ANOVA with Tukey test for multiple comparisons between each parameter for all strains and each drug): figures with different lower case letters (analysis per column) or upper case letters (analysis per row) displayed below values in the table are significantly different from each other (P<0.05).

[c]Increase in cfu (in $\log_{10}$ units) from the corresponding original inoculum as extrapolated for infinitely low concentration of antibiotics (mean with 95% CI).

[d]Decrease in cfu (in $\log_{10}$ units) from the corresponding original inoculum as extrapolated for infinitely large concentration of antibiotics (mean with 95% CI).

[e]Concentration (mg/L) resulting in no apparent bacterial growth as determined by graphical interpolation. MIC values used: values measured at 24 h and pH 5.5 (in the presence of haemin for the *hemB* mutant, based on previous data suggesting availability of haemin-like compounds in the cellular medium[1]).

Table S2. Pertinent regression parameters[a] (with CIs) and statistical analysis[b] of the dose-response curves illustrated in Figure S2

	Control conditions						+NAC					
				C_s[e]						C_s[e]		
Antibiotic	strain	E_{min}[c]	E_{max}[d]	mg/L	× MIC (pH 5.5)	R^2	strain	E_{min}[c]	E_{max}[d]	mg/L	× MIC (pH 5.5)	R^2
Cloxacillin	OM1a	3.73 (3.20 to 4.27) a,A	-1.04 (-1.48 to -0.59) a,A	1.53	6.12	0.982	OM1a	3.87 (3.12 to 4.62) a,A	-0.91 (-1.49 to -0.34) a,A	1.41	5.64	0.968
	OM1b	2.92 (2.31 to 3.53) b,A	-1.17 (-1.39 to -0.94) a,A	0.06	0.48	0.984	OM1b	3.88 (3.35 to 4.42) a,B	-1.14 (-1.60 to -0.68) a,A	1.62	13.0	0.984
	OM1bs	4.01 (3.69 to 4.34) a,A	-1.19 (-1.36 to -1.02) a,A	0.29	1.16	0.996	OM1bs	3.75 (3.09 to 4.42) a,A	-1.08 (-1.69 to -0.47) a,A	2.09	8.36	0.971
Doripenem	OM1a	4.28 (3.83 to 4.72) a,A	-0.850 (-1.06 to -0.64) a,A	0.30	10	0.991	OM1a	4.16 (3.46 to 4.86) a,A	-0.85 (-1.24 to -0.47) a,A	0.52	17.3	0.979
	OM1b	4.80 (2.63 to 6.97) a,A	-1.08 (-1.24 to -0.92) a,A	0.01	0.33	0.983	OM1b	4.38 (3.90 to 4.85) a,A	-0.95 (-1.20 to -0.71) a,b,A	0.37	12.3	0.992
	OM1bs	4.20 (3.40 to 5.00) a,A	-0.76 (-1.00 to -0.52) a,A	0.08	2.67	0.979	OM1bs	4.22 (3.72 to 4.71) a,A	-1.20 (-1.46 to -0.93) b,B	0.32	10.7	0.991
Meropenem	OM1a	4.53 (4.09 to 4.98) a,A	-1.04 (-1.26 to -0.82) a,A	0.30	2.40	0.994	OM1a	4.55 (3.65 to 5.44) a,A	-0.53 (-0.98 to -0.08) a,B	0.59	4.72	0.969
	OM1b	2.71 (2.01 to 3.40) b,A	-1.16 (-1.35 to -0.97) a,A	0.02	0.33	0.981	OM1b	4.13 (3.18 to 5.08) a,B	-0.49 (-0.98 to -0.00) a,B	0.63	10.5	0.958
	OM1bs	4.08 (3.27 to 4.89) a,A	-1.19 (-1.62 to -0.76) a,A	0.30	2.40	0.976	OM1bs	4.16 (3.08 to 5.25) a,A	-0.48 (-1.00 to 0.037) a,B	0.50	4.00	0.950

Vancomycin	OM1a	3.75 (3.33 to 4.17) a,A	-1.15 (-1.63 to -0.68) a,A	5.01	5.01	0.986	OM1a	4.20 (3.82 to 4.57) a,A	-0.85 (-1.23 to -0.47) a,A	4.68	4.68	0.990
	OM1b	0.82 (0.62 to 1.02) b,A	-1.17 (-1.38 to -0.96) a,A	0.72	1.44	0.982	OM1b	4.08 (3.42 to 4.74) a,B	-1.40 (-1.99 to -0.82) a,A	1.55	3.10	0.978
	OM1bs	3.63 (2.87 to 4.40) a,A	-1.34 (-2.18 to -0.50) a,A	3.80	7.60	0.956	OM1bs	4.23 (3.78 to 4.68) a,B	-1.45 (-1.86 to -1.04) b,A	1.73	3.46	0.990

[a]Calculated based on sigmoidal regressions with an Hill coefficient of 1 for extracellular data and for intracellular data with cloxacillin, doripenem, meropenem and vancomycin.

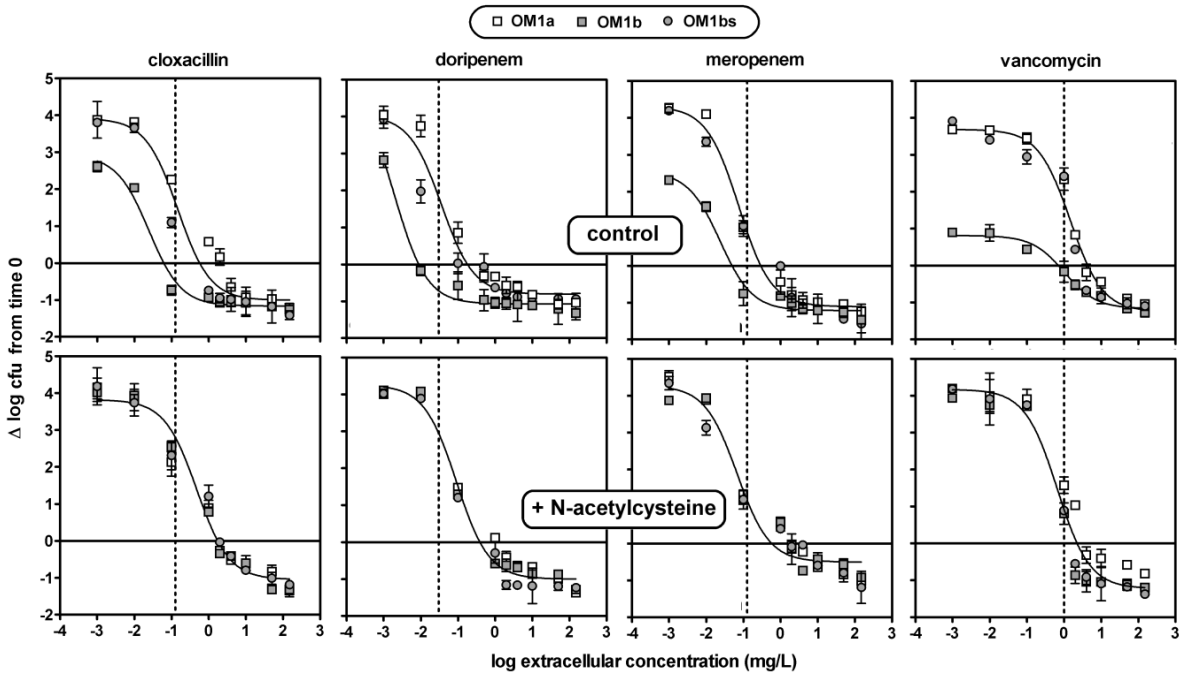
[b]Statistical analyses: (one-way ANOVA with Tukey test for multiple comparisons between each parameter for all strains and each drug): figures with different lower case letters (analysis per column) or upper case letters (analysis per row) displayed below values in the table are significantly different from each other ($P<0.05$).

[c]Increase in cfu (in $\log_{10}$ units) from the corresponding original inoculum as extrapolated for infinitely low concentration of antibiotics (mean with 95% CI).

[d]Decrease in cfu (in $\log_{10}$ units) from the corresponding original inoculum as extrapolated for infinitely large concentration of antibiotics (mean with 95% CI).

[e]Concentration (mg/L) resulting in no apparent bacterial growth as determined by graphical interpolation. MIC values used: values measured at 24 h and pH 5.5 (in the presence of haemin for the *hemB* mutant, based on previous data suggesting availability of haemin-like compounds in the cellular medium[1]).

Figure S1. Concentration-response curves of cloxacillin, doripenem, meropenem and vancomycin against the intracellular forms of isogenic strains of *S. aureus* with an MSSA phenotype [wild-type (OM1a) and *menD* mutant in control conditions (OM1b) or in medium supplemented with 2 mg/L MSB (OM1bs)]. Infected cells were incubated in the presence of increasing concentrations of antibiotics (total drug) for 24 h in control conditions (top panels) or in the presence of 25 mM NAC (bottom panels). The ordinate shows the change in the number of cfu (log scale) per mg of cell protein. The continuous horizontal line corresponds to an apparent static effect. The abscissa shows the drug extracellular concentration. The broken vertical line corresponds to the MIC for the parental strain as measured at pH 5.5 (24 h reading). All values are means±SD of three independent determinations (when not visible, the SD bars are smaller than the size of the symbols). Experiments have been reproduced three times with similar results. For sake of clarity, individual curves are shown only for strains with data significantly differing (P<0.05) from one another by repeated measures ANOVA followed by Tukey's multiple comparison test. When not statistically different, data from different strains were analysed together to calculate a single Hill function. Individual regression parameters and pharmacological descriptors are shown in Table S2.



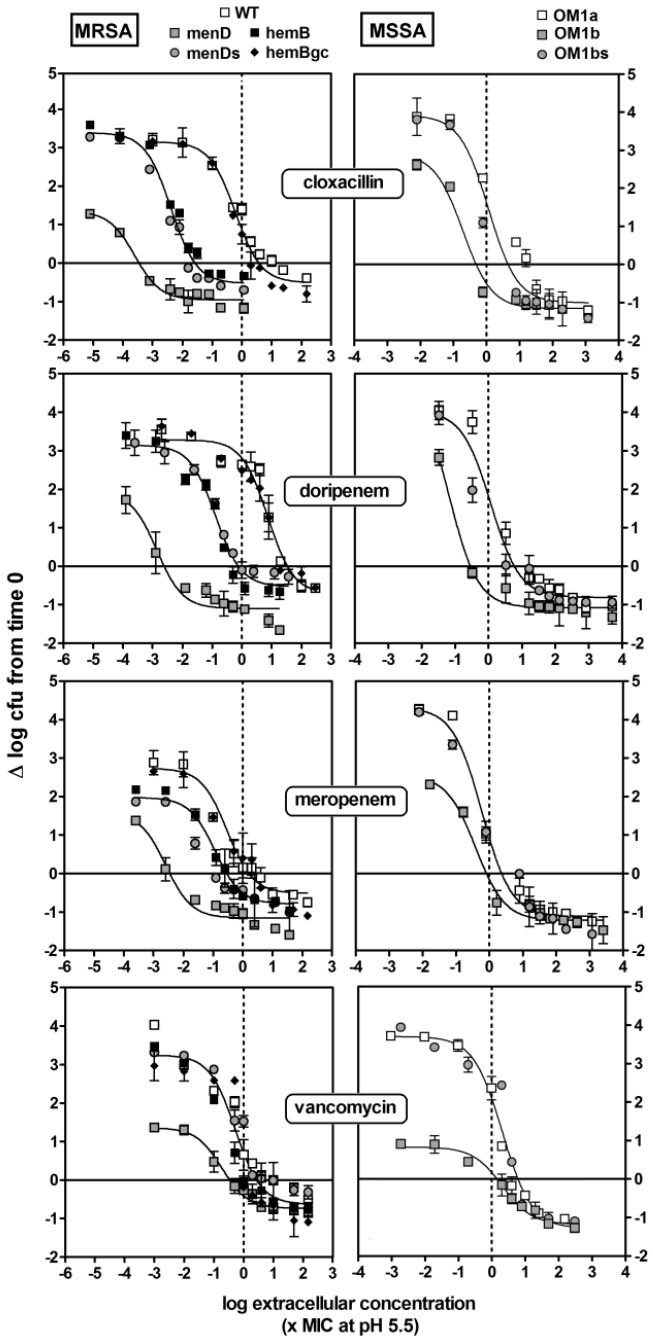
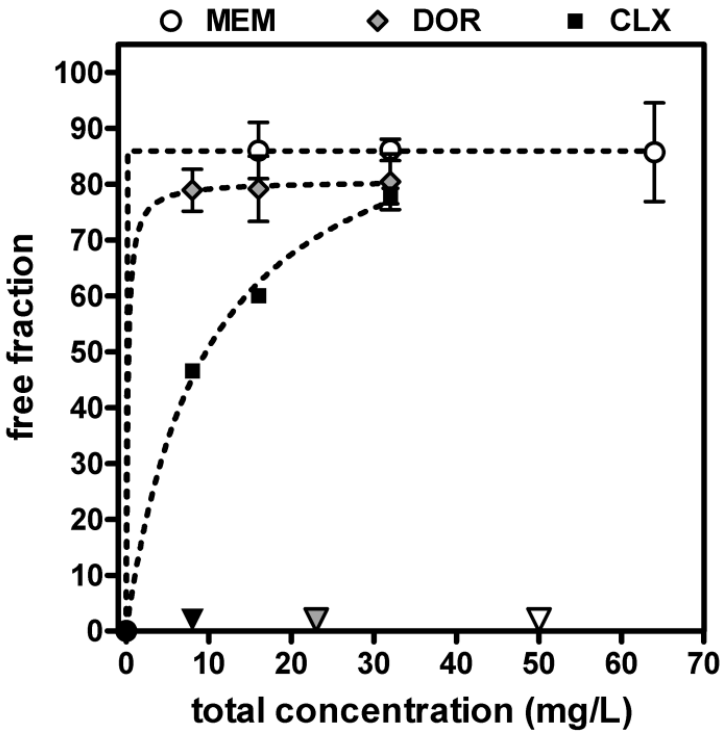


Figure S2. Concentration-response curves of cloxacillin, doripenem, meropenem and vancomycin against the intracellular forms of isogenic strains of *S. aureus* with an MRSA phenotype [left-hand panels; wild-type (WT), *menD* mutant in control conditions (menD) or in medium supplemented with 2 mg/L MSB (menDs), *hemB* mutant (hemB) and the *hemB* genetically complemented mutant (hemBgc)] or an MSSA phenotype [right-hand panels; wild-type (OM1a) and *menD* mutant in control conditions (OM1b) or in medium supplemented with 2 mg/L MSB (OM1bs)]. Infected cells were incubated in the presence of increasing concentrations of antibiotics for 24 h. The ordinate shows the change in the number of cfu (log scale) per mg of cell protein. The continuous horizontal line corresponds to an apparent static effect. The abscissa shows the drug extracellular concentration expressed in × MIC measured at pH 5.5 (except the *hemB* strain) or pH 5.5 in the presence of haemin (the *hemB* strain, based on previous data suggesting availability of haemin-like compounds in the cellular medium[1]). All values are means±SD of three independent determinations (when not visible, the SD bars are smaller than the size of the symbols). For sake of clarity, individual curves are shown only for strains with data significantly differing (P<0.05) from one another by repeated measures ANOVA followed by Tukey's multiple comparison test. When not statistically different, data from different strains were analysed together to calculate a single Hill function.

Figure S3. Free concentration of β-lactams in RPMI-1640 medium supplemented with 10% fetal bovine serum, after 2 h of incubation at 37°C. Values are means±SD of three independent determinations (when not visible, the SD bars are smaller than the size of the symbols). The triangles on the *x*-axis point to the respective human C_{max} (total drug) for these drugs with conventional dosages [cloxacillin (CLX, black), 8 mg/L; doripenem (DOR, grey), 23 mg/L; and meropenem (MER, white), 50 mg/L].



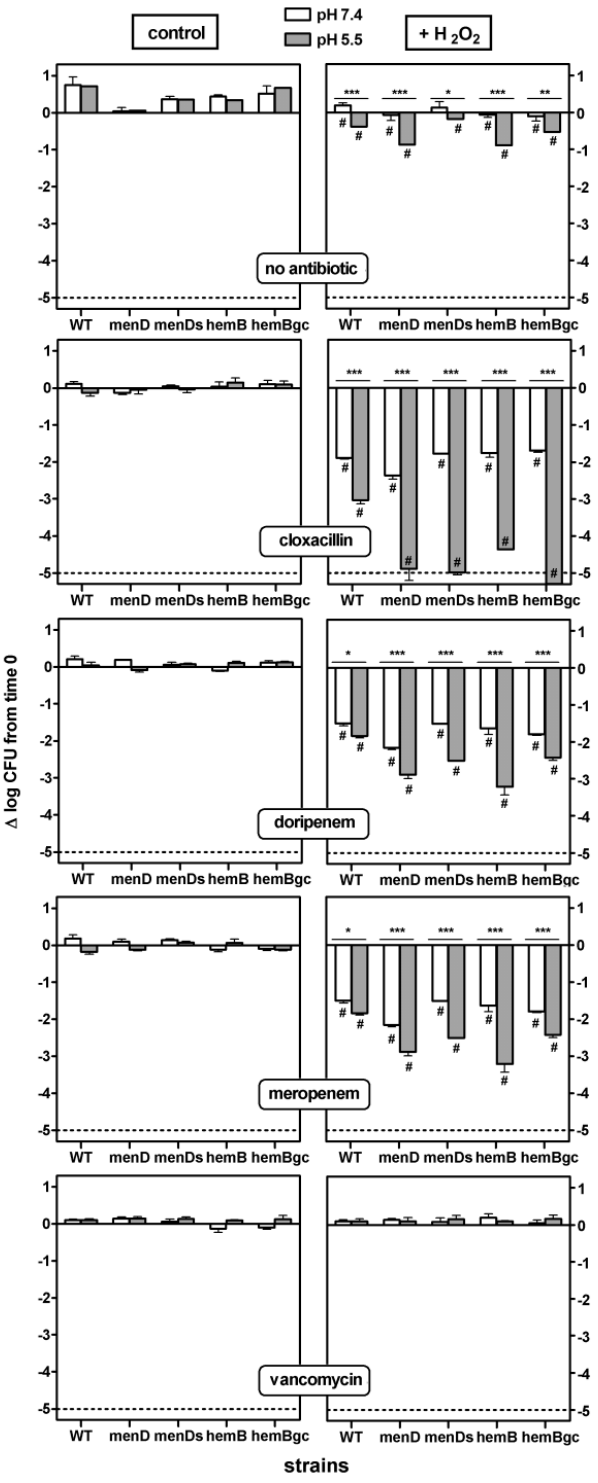


Figure S4. Influence of pH and H_2O_2 on the activity of antibiotics against the isogenic strains of *S. aureus* with an MRSA phenotype [wild-type (WT), *menD* mutant in control conditions (menD) or in medium supplemented with 2 mg/L MSB (menDs), *hemB* mutant (hemB) and the *hemB* genetically complemented mutant (hemBgc)]. Bacteria were pre-exposed (right-hand panels) or not (left-hand panels) to 10 mM H_2O_2 for 30 min, then incubated in broth at pH 7.4 (white bars) or 5.5 (grey bars) in the presence of antibiotics for 5 h at concentrations equivalent to their MIC at the corresponding pH. The ordinate shows the change in bacterial counts from the initial inoculum. The continuous horizontal line corresponds to an apparent static effect and the broken horizontal line corresponds to the limit of detection. All values are means±SD of three independent determinations (when not visible, the SD bars are smaller than the size of the symbols). Experiments have been reproduced two times with similar results. Statistical analysis (ANOVA with Tukey post-hoc test): comparisons between pH 7.4 and pH 5.5 for each individual strain (**P*<0.05, ***P*<0.01 and ****P*<0.001); and comparisons between control conditions and preincubation with H_2O_2 for each individual strain at a given pH (#*P*<0.05).

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