Penicillin-binding protein PBP2a provides variable levels of protection toward different β-lactams in Staphylococcus aureus RN4220

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Abstract
Methicillin-resistant Staphylococcus aureus (MRSA) is resistant to most β-lactams due to the expression of an extra penicillin-binding protein, PBP2a, with low β-lactam affinity. It has long been known that heterologous expression of the PBP2a-encoding meCA gene in methicillin-sensitive S. aureus (MSSA) provides protection towards β-lactams, however, some reports suggest that the degree of protection can vary between different β-lactams. To test this more systematically, we introduced an IPTG-inducible meCA into the MSSA laboratory strain RN4220. We confirm, by growth assays as well as single-cell microfluidics time-lapse microscopy experiments, that PBP2a expression protects against β-lactams in S. aureus RN4220. By testing a panel of ten different β-lactams, we conclude that there is also a great variation in the level of protection conferred by PBP2a. Expression of PBP2a resulted in an only fourfold increase in minimum inhibitory concentration (MIC) for imipenem, while a 32-fold increase in MIC was observed for cefaclor and cephalexin. Interestingly, in our experimental setup, PBP2a confers the highest protection against cefaclor and cephalexin—two β-lactams that are known to have a high specific affinity toward the transpeptidase PBP3 of S. aureus. Notably, using a single-cell microfluidics setup we demonstrate a considerable phenotypic variation between cells upon β-lactam exposure and show that meCA-expressing S. aureus can survive β-lactam concentrations much higher than the minimal inhibitory concentrations. We discuss possible explanations and implications of these results including important aspects regarding treatment of infection.

Keywords
meca, microfluidics, MRSA, time-lapse microscopy, β-lactams
1 | INTRODUCTION

Staphylococcus aureus is a major pathogen responsible for a range of different infections in both animals and humans, including skin and wound infections, mastitis, and bacteremia. Besides, both animals and humans can be asymptomatic carriers of these bacteria. Traditionally, β-lactams alone or in combination with other substances have been used successfully to treat staphylococcal infections, due to their low toxicity, good pharmacodynamics, and bactericidal action (Foster, 2019; Llarrull, Fisher, & Mobashery, 2009). However, the spread of β-lactam resistant staphylococcal strains has emerged as a global concern (Grundmann, Aires-de-Sousa, Boyce, & Tiemersma, 2006), making it increasingly difficult to combat these infections.

β-lactam antibiotics function by inhibiting the transpeptidase activity of penicillin-binding proteins (PBPs). PBPs are essential for the last steps of the synthesis of peptidoglycan in the bacterial cell wall. Peptidoglycan consists of glucan chains with alternating N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM) units, which are cross-linked by peptide bridges between the stem peptides of the NAMs units (Egan, Cleverley, Peters, Lewis, & Vollmer, 2017; Typas, Banzhof, Gross, & Vollmer, 2011). Synthesis of peptidoglycan requires two enzymatic reactions: NAGs and NAMs are added to the growing peptidoglycan chain by transglycosylases, and the cross-links are formed by transpeptidases (Lovering, Safadi, & Strynadka, 2012; Typas et al., 2011). β-lactams mimic the D-alan-D-alanine residues on the NAM side chain and form a covalent bond to a serine residue in the transpeptidase active site to inhibit PBP activity (Peacock & Paterson, 2015).

Staphylococcus aureus encodes four different PBPs, named PBP1-4 (Pinho, Kjos, & Veening, 2013) which can be targeted by β-lactams. Two of these, PBP1 and PBP3, are monofunctional transpeptidases, meaning that they only catalyze the formation of peptide crossbridges. PBP1 and PBP3 interact with proteins of the SEED (shape, elongation, division, and sporulation) family (FtsW and RodA, respectively) to form active transpeptidase/transglycosylase pairs (Meeske et al., 2016; Reichmann et al., 2019). In contrast to PBP1 and PBP3, the PBP2 is a bifunctional protein with both transpeptidase and transglycosylase activities in the same protein. The last PBP, PBP4 is a nonessential low-molecular-weight PBP with transpeptidase activity, whose function is still to a large extent undefined (da Costa, de Oliveira, Chambers, & Chatterjee, 2018).

Methicillin-resistant S. aureus (MRSA) strains encode, in addition to PBP1-4, a fifth PBP protein, known as PBP2a, which is responsible for the resistant phenotype. Even though methicillin is no longer in use, the term methicillin resistance persists and represents resistance to practically all β-lactams, except 5th generation cephalosporins (Peacock & Paterson, 2015). PBP2a is encoded by the mecA gene located on a genomic island known as staphylococcal cassette chromosome mec (SCCmec) (Katayama, Ito, & Hiramatsu, 2000). PBP2a is a transpeptidase with a reduced affinity for transpeptidase-inhibiting β-lactams. This low affinity allows MRSA strains to continue cell wall synthesis and multiplication in the presence of β-lactams, as the transpeptidase activity of PBP2a is still functional when the activities of the other PBPs are inhibited.

PBP2a activity in MRSA is regulated on many levels. For example, PBP2a is under allosteric control (Fuda et al., 2005; Otero et al., 2013). Correct folding and activity of PBP2a are also known to be dependent on extracellular chaperones (Jousselin et al., 2015; Roch et al., 2019), while mecA transcription is influenced by several factors (Hao, Dai, Wang, Huang, & Yuan, 2012; Peacock & Paterson, 2015). Importantly, the stringent stress response pathway, specifically mediated by changes in the guanine metabolism, is associated with high-level β-lactam resistance in MRSA strains (Kim et al., 2013; Mwangi et al., 2013; Tomasz, Nachman, & Leaf, 1991). Many MRSA strains also display so-called heterogeneous resistance where only a fraction of the cells in a population are resistant (de Lencastre & Tomasz, 1994; Tomasz et al., 1991). Induction of the stringent stress response can change this heterogeneous resistant phenotype to a homogeneous, high-level β-lactam resistant phenotype (Aedo & Tomasz, 2016).

Different β-lactam subclasses, such as penicillins, cephalosporins, carbapenems, and monobactams, all have the β-lactam ring as the functional core. Apart from that, they contain chemical features which give different properties, such as different sensitivities toward β-lactamases (Bush, 2018) and selective affinities for different PBPs (Chambers, Sachdeva, & Kennedy, 1990; Georgopapadakou, Smith, & Bonner, 1982; Kocaoglu, Tsui, Winkler, & Carlson, 2015). Such detailed knowledge about the characteristics of these antibiotics and their interplay with bacteria could be utilized and explored in the design of individually tailored treatment schemes of difficult-to-treat infections. Due to the increasing spread and treatment challenges of MRSA, it is necessary to gain further insight into the β-lactam resistance of S. aureus. In this work, we investigated how PBP2a protected against different β-lactams in when expressed in the MSSA-strain S. aureus RN4220.

2 | RESULTS AND DISCUSSION

2.1 | Heterologous expression of mecA in S. aureus RN4220 results in dose-dependent cefoxitin resistance

Heterologous expression of mecA has previously been shown to confer resistance to β-lactams in S. aureus MSSA strains (Ballhausen, Kriegeskorte, Schleimer, Peters, & Becker, 2014; Matthews, Reed, & Stewart, 1987; Murakami & Tomasz, 1989). We introduced the PBP2a-encoding gene mecA downstream of the Pspac promoter on a plasmid in the MSSA laboratory strain RN4220 (pLOW-mecA, strain MF7). This strain shows controlled mecA expression from the well-established pLOW plasmid (Liew et al., 2011) by the addition of increasing concentrations of IPTG. Expression of mecA did not influence the growth of the resulting strain; no growth defect was observed in MF7 (Pspac-mecA) compared to the control strain (vector control strain IM55 carrying pLOW without mecA) for any of the
inducer concentrations (Figure 1a). Ender, McCallum, Adhikari, and Berger-Bächi (2004) found that transformation of a type I SCCmec element into a naïve susceptible strain resulted in a slower growth rate compared to the parental strain. However, with our experimental conditions, there was no apparent fitness cost related to heterogeneous expression of the mecA gene in an MSSA.

To first establish that mecA expression could confer resistance in RN4220 under our experimental conditions, we exposed the cells to cefoxitin, a cephalosporin commonly used to detect MRSA strains (Skov et al., 2006, 2014). As expected, mecA induction protected S. aureus against cefoxitin (Figure 1b–f). The MF7 strain (Pspac–mecA) grown with a range of inducer concentrations (0–1,000 µM IPTG) was exposed to twofold dilution series of cefoxitin to determine the minimum inhibitory concentrations (MIC). The MIC of the uninduced strain was 1 µg/ml (Table 1), and 50 µM IPTG was needed to increase the MIC to cefoxitin twofold (Figure 1a). By further increasing the inducer concentration, a maximum of eightfold increase in MIC was obtained compared to the noninduced MF7 strain (Figure 1d,e).

**FIGURE 1** Growth of *Staphylococcus aureus* RN4220 expressing mecA. (a) Expression of mecA, mecA(S403A) or mecA(K188A) in *S. aureus* does not affect growth. Growth of MF7 (Pspac–mecA), MF21 (Pspac–mecA(S403A)), and MF23 (Pspac–mecA(K188A)) with maximum mecA induction (1,000 µM IPTG) were compared to growth of uninduced MF7 and a vector control strain. (b–f) Growth curves of MF7 showing the effect of cefoxitin on growth with different mecA induction levels. For each inducer concentration, growth curves in the presence of five different cefoxitin concentrations are shown (0, 2, 4, 8, 16 µg/ml). (b) No mecA induction, (c) 10 µM IPTG, (d) 50 µM IPTG, (e) 250 µM IPTG, and (f) 1,000 µM IPTG. (g) Population analysis profile of cefoxitin for MF7 (Pspac–mecA) and control strain induced with 250 µM IPTG. The strains were plated onto plates with different concentrations of cefoxitin. The population analysis profile shows that MF7 has a heterogeneous resistant phenotype.
our experiments (Table 2). Thus, heterologous expression of PBP2a confers protection toward cefoxitin in *S. aureus* RN4220.

PBP2a has also been shown to be under allosteric control (Fuda et al., 2005; Otero et al., 2013), however, it has not been studied whether the allostery of PBP2a plays any role during heterologous expression in an MSSA strain. To see if allosteric regulation played a role under our experimental conditions, we therefore created another mutant, mecA(K188A) (strain MF23), where one of the key residues for allosteric regulation was mutated (Otero et al., 2013). However, this mutation did not have any effect on the activity of PBP2a and the mutant was as efficient as the wild-type PBP2a protein in protecting against the different β-lactams (Table 2). Thus, based on the results of this mutant, allosteric regulation does not seem to play any role in our experimental setup.

### 2.2 Variable levels of protection toward different β-lactams by PBP2a in *S. aureus* RN4220

The results above establish that the P_{spec-mecA} construct in *S. aureus* RN4220 can protect the cells against cefoxitin up to eightfold compared to the controls. Previously, studies have indicated that expression of mecA in an MSSA background may confer variable levels of protection against different β-lactams (Ballhausen et al., 2014; Ubukata, Nonoguchi, Matsumishi, & Konno, 1989). To study this variation more systematically, we tested the resistance levels of strain MF7 toward a panel of 10 different β-lactams. These represent ten different β-lactam subclasses, including penicillins (1st–4th generation), carbapenems, and cephalosporins (1st–3rd generation) (Table 1). Furthermore, it has long been known that different β-lactams have variable affinities for the four native staphylococcal PBPs (Chambers & Sachdeva, 1990; Chambers et al., 1990; Georgopapadakou, Dix, & Mauriz, 1986; Georgopapadakou, Smith, Cimarusti, & Sykes, 1983), and we included β-lactams with variable affinity characteristics: Cefoxitin has the highest affinity for PBP4, cefotaxime has the highest affinity for PBP2 and cephalaxin and cefaclor has the highest affinity for PBP3 (Chambers et al., 1990; Georgopapadakou et al., 1982, 1986). The other β-lactams tested also have variable but less defined affinities for PBPs (Baller et al., 1986).

MICs were determined in strain MF7 with or without induction of mecA by IPTG (Table 1). Notably, the level of protection for the different β-lactams ranged from fourfold to a 32-fold increase in MIC from induced to uninduced. Cefaclor and cephalaxin showed the highest increase in MIC (32-fold), followed by ampicillin and oxacillin (16-fold). The lowest MIC increase was found for imipenem (fourfold) followed by cefotaxime, cefoxitin, penicillin G, and piperacillin (all eightfold). These results strengthen and underline previous indications (Ballhausen et al., 2014; Ubukata et al., 1989) that there is a great variation in the level of β-lactam protection conferred by mecA upon expression in an *S. aureus* MSSA strain. The observed variations do not seem to correlate with β-lactam subclasses (i.e., penicillins vs cephalosporins, Table 1). The highest level (32-fold) of protection was found against cefaclor and cephalaxin. Notably, these are β-lactams that are characterized by having high specific affinity toward PBP3 (Chambers & Sachdeva, 1990; Georgopapadakou et al., 1982). On the other hand, the two other selective β-lactams tested, cefotaxime and cefoxitin, which have the highest affinity toward PBP2 and PBP4, respectively (Chambers & Sachdeva, 1990), showed clearly lower resistance levels (both eightfold resistance).

To further study the notable observation that the highest level of protection was conferred against PBP3-selective β-lactams in our
TABLE 2 MIC values and level of protection against antibiotics with PBP selectivity with a gradual increase in mecA expression in Staphylococcus aureus MF7

| ABX        | Strain | Genotype         | Concentration IPTG (µM) | Fold protectionb |
|------------|--------|------------------|-------------------------|-----------------|
|            |        |                  | 0           | 50     | 250     | 1,000   |                  |
| Cefoxitin  | IM55   | Control          | 1           | 1      | 1       | 1       | 1                |
| MF7        |        | Pspac mecA       | 1           | 2      | 4       | 8       | 8                |
| MF21       | Pspac mecA (S403A) | 1           | 1      | 1       | 1       | 1                |
| MF23       | Pspac mecA (K188A) | 1           | 2      | 8       | 8       | 8                |
| Cefotaxime | IM55   | Control          | 0.5         | 0.5    | 0.5     | 0.5     | 1                |
| MF7        |        | Pspac mecA       | 0.5         | 2      | 4       | 4       | 8                |
| MF21       | Pspac mecA (S403A) | 0.5         | 0.5    | 0.5     | 0.5     | 1                |
| MF23       | Pspac mecA (K188A) | 0.5         | 1      | 4       | 4       | 8                |
| Cefaclor   | IM55   | Control          | 0.5         | 0.5    | 0.5     | 0.5     | 1                |
| MF7        |        | Pspac mecA       | 0.5         | 4      | 16      | 16      | 32               |
| MF21       | Pspac mecA (S403A) | 0.5         | 0.5    | 0.5     | 0.5     | 1                |
| MF23       | Pspac mecA (K188A) | 1           | 4      | 16      | 16      | 32               |
| Cephalexin | IM55   | Control          | 2           | 2      | 2       | 2       | 1                |
| MF7        |        | Pspac mecA       | 2           | 2      | 2       | 2       | 1                |
| MF21       | Pspac mecA (S403A) | 2           | 8      | 64      | 64      | 32               |
| MF23       | Pspac mecA (K188A) | 2           | 2      | 2       | 2       | 1                |

aThe experiments were repeated at least three times with similar results.
bFold protection by mecA induction was determined as the ratio between MIC with 1,000 µM induction and the MIC of uninduced cells.

experiments, we made an IPTG titration with concentrations from 0 to 1,000 µM and again determined the MICs for these PBP-selective β-lactams (Table 2). This further demonstrated that PBP2a is more effective in protecting against cefaclor and cephalexin than against cefoxitin and cefotaxime (Table 2) at different PBP2a expression levels. In all cases, the MIC value of the uninduced MF7 was similar to the wild type and control strain, verifying the leaky expression of mecA did not play any significant role (Table 1, Table 2). In line with what was found for cefoxitin, the active site mutant did not confer resistance to any of the β-lactams and we found no indication that allosteric regulation plays a role in these assays since the mecA(K188A) mutant provided a similar degree of protection as the original mecA allele.

PBP3 is a monofunctional, nonessential transpeptidase that is thought to be important for the slight cell elongation observed during the staphylococcal cell cycle (Reichmann et al., 2019). PBP2a, which is also a transpeptidase, replaces the transpeptidase activity of PBP2 in MRSA strains, but cannot complement the transpeptidase activity of PBP1 (Pereira, Henriques, Pinho, de Lencastre, & Tomasz, 2007; Pinho, de Lencastre, & Tomasz, 2001). It is possible that PBP2a functionally complements the β-lactam-inhibited PBP3 activity and that this somehow contributes to the observation that PBP2a expression confers the highest level of resistance toward PBP3-selective β-lactams. This trend has not been reported before. In contrast, Antignac and Tomasz (2009) compared the MICs of the homogeneously resistant MRSA strain COL with an isogenic strain in which mecA was deleted (COL-S) and found that the drop in resistance levels was more pronounced for PBP2- and PBP4-specific β-lactams as compared to a PBP3-specific β-lactam (Antignac & Tomasz, 2009; Georgopapadakou et al., 1982). It can be speculated that these differences may be due to strain-specific host factors important for optimal resistance (Berger-Bächi & Rohrer, 2002; Roemer, Schneider, & Pinho, 2013), for example, proteins affecting the cell wall synthesis machinery. However, to shed further light on the mechanisms underlying the observed variation in PBP2a-mediated protection against different β-lactams in S. aureus RN4220 observed here, future studies should systematically compare mecA expression in S. aureus MSSA strains with different genetic backgrounds.

2.3 | Heterologous mecA expression confers a low level of protection against β-lactams

In addition to the β-lactam-dependent variation, the results presented in Table 1 also show that the level of protection conferred by mecA expression in S. aureus RN4220 is relatively low. Upon full induction of mecA, the MICs for cefoxitin and cephalexin were 8 and 64 µg/ml, respectively. Both these MICs are significantly lower than those for MRSA strains carrying the full SCCmec. We determined the MICs for cefoxitin and cephalexin for the homogeneous resistant MRSA strain S. aureus COL and found these to be 188 and 125 µg/ml, respectively. The relatively low MICs in RN4220 upon heterologous mecA expression is in line with a study by Ballhausen et al. (2014) where mecC and mecA expression in RN4220 resulted in cefoxitin resistance levels in the same range as observed here. These differences between MRSA strains and heterologous expression of mecA
in MSSA strains may be explained by the variable transcription level of PBP2a between strains. Alternatively, unknown strain-dependent factors may play an important role. For example, the functionality of PBP2a is likely to be better adapted and optimized in their natural MRSA hosts compared to non-native expression in MSSA hosts such as RN4220 (Katayama et al., 2003). This notion may also be one of several reasons for the limited horizontal gene transfer events observed for mecA in S. aureus (Peacock & Paterson, 2015) since the competitive advantage of strains with newly acquired mecA is relatively low upon exposure to high doses of β-lactams.

2.4 | Single-cell analysis of heterologous mecA expression

To further observe how heterologous mecA expression protects the RN4220 cells toward β-lactams on a single-cell level, we performed microfluidics fluorescence time-lapse microscopy. To allow cocultivation experiments, we created a GFP-positive RN4220 strain by integrating a gfp gene on the RN4220 chromosome, following a previously published approach (de Jong, van der Horst, van Strijp, & Nijland, 2017). The P_{spac}-mecA(S403A) construct, expressing the nonactive PBP2A, was transformed into the GFP-positive strain to create strain MF27. As expected, the MICs of strain MF27 were shown to be identical to those of MF21 and wild-type RN4220 (data not shown). The single-cell growth of the two strains MF7 (P_{spac}-mecA) and MF27 (P_{spac}-mecA(S403A), GFP+) was then studied in culture medium without cefoxitin and in the presence of 2 and 20 μg/ml cefoxitin. These concentrations correspond to twofold and 20-fold higher than the MIC for S. aureus RN4220 (Table 1). Expression from the P_{spac} promoter was induced throughout the experiment. In the absence of cefoxitin (Figure 2a), the two strains were both actively multiplying. At the lower cefoxitin concentration (Figure 2b, 2 μg/ml), MF7 was growing normally, while the MF27 strain stopped dividing. In the presence of 20 μg/ml, which is more than 20× MIC of MF27 and more than 2× MIC of MF7, neither of the strains were multiplying (Figure 2c). Thus, these single-cell data are fully in line with the growth curves.

It was interesting to note, however, that only a fraction of the mecA-negative cells (MF27) lysed, as observed by loss of GFP-signal upon exposure to cefoxitin. During the 6 hr timeframe of the experiments, exposure to 2× MIC (2 μg/ml cefoxitin) resulted in lysis of 17.3% (N = 243) of the cells. The fraction increased somewhat upon exposure to 20× MIC of cefoxitin (28.1% of cell lost GFP-signal, N = 238). Still, the majority of cells even at this concentration (20× MIC of MF27) did not lyse. Our observations thus show that there are cell-to-cell variations with regard to cell lysis upon cefoxitin exposure.

To see whether this was a cefoxitin-specific phenotype, the same type of experiments was then performed with cefotaxime, a 3rd generation cephalosporin. The MIC of the control strain for cefotaxime is 0.5 μg/ml, and similar to cefoxitin, induction of mecA expression resulted in an eightfold increase in MIC (Table 1). During the time-lapse microscopy, cells were exposed to 2 μg/ml (4× MIC of control) and 20 μg/ml (40× MIC of control) for four hours. In these experiments, we changed to cefotaxime-free medium after four hours to study the potential recovery and regrowth of cells after antibiotic exposure. As for cefoxitin, only a small fraction of the control cell (MF27) lysed during four hours (Figure 3, 8.1% and 7.6%, respectively). However, upon changing to normal growth medium after four hours of cefotaxime exposure, the MF27 cells did not regrow. This shows, as expected, that all the cells were killed by the bactericidal β-lactam although only a fraction of the cells lysed. It is well established that β-lactams inhibit the PBP transpeptidase activity, however, the exact mechanism leading to cell killing by β-lactams is still

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**FIGURE 2** Microfluidics fluorescence microscopy time-lapse experiments in the presence of different concentrations of cefoxitin. The two strains MF7 (P_{spac}-mecA, dark cells) and MF27 (P_{spac}-mecA(S403A), GFP+ green cells) were mixed in equal ratios, and pregrown in media with 250 μM IPTG to induce expression of the mecA alleles. Single-cell growth was analyzed in medium (a) without cefoxitin and in the presence of (b) 2 μg/ml and (c) 20 μg/ml cefoxitin using a CellASIC ONIX Microfluidics setup. 250 μM IPTG was present in all conditions to induce the expression of mecA alleles. White arrowheads point to lysing cells. See also Movies S1–S3 (https://doi.org/10.6084/m9.figshare.12168351.v1)
not fully understood (Peacock & Paterson, 2015). Cell wall degrading enzymes seem to have variable importance in different strains and for different β-lactams (Peacock & Paterson, 2015). The cell-to-cell variation observed here with regard to lysis thus suggests that several mechanisms resulting in cell killing at play in the same population.

The growth of mecA-positive MF7-cells was, as expected, fully inhibited at the highest cefotaxime concentration (20 µg/ml) (Figure 2b). This concentration is fivefold higher than cefotaxime MIC after mecA induction (Table 2). Noteworthy, however, upon changing to normal growth medium after four hours of cefotaxime exposure, some of these cells (13%, N = 170) were able to regrow after the antibiotic exposure was released (Figure 3, yellow arrowheads). The presence of PBP2a in these cells thus protected the cells from β-lactam-mediated killing even with four hours exposure with concentration much higher than the MIC value. The reason for the cell-to-cell variation is not known. As shown in Figure 1g, the MF7 strain has a heterogeneous resistant phenotype and this result underlines the importance of prolonged drug treatment to kill all cells in a population.

### 3 | CONCLUSIONS

The results presented here show (a) that the level of resistance conferred by mecA expression in *S. aureus* RN4220 is low compared to MRSA strains, and that (b) the level of resistance varies considerably between different β-lactams. Surprisingly, and in contrast to what has been reported for MRSA strains, the highest level of resistance is observed for PBP3-targeting β-lactams. The reason for this is unknown and should be subjected to further studies. It is not known whether these variable MICs observed here would be valid in clinical isolates of MRSA. However, it underlines the importance of determining the MIC for the specific antibiotic toward the individual pathogenic strain when preparing for the treatment of an MRSA infection, as well as considering the achievable drug concentration at the site of infection.

Our experiments were done in a laboratory strain with heterologous expression of mecA. If the observations reported here also are representative for wild-type populations of *S. aureus*, this will pose critical problems for diagnostics and treatment of such infections. For example for cefoxitin, the MIC of resistant isolates based on the Clinical and Laboratory Standards Institute (CLSI) guidelines is >8 µg/ml (CLSI, 2020), and wild-type MRSA strains often show MICs more than 10-fold higher (e.g., 188 µg/ml for *S. aureus COL*). Due to the relatively low MIC values after mecA induction (e.g., 8 µg/ml for cefoxitin), such a strain could be interpreted as intermediate or even negative for methicillin resistance. Besides, the single-cell data suggest that some cells can survive at concentrations fivefold higher than the MIC and thus also regrow when no longer exposed to antibiotics. This is a critical aspect in the treatment of infections and further highlights the importance of achievable drug concentrations and duration of drug exposure, to prevent such survivor cells to regrow and avoid re-emergence of infections.

### 4 | MATERIALS AND METHODS

#### 4.1 | Bacterial strains and growth conditions

All strains used in this study are listed in Table 3. *Escherichia coli* was grown in LB medium at 37°C with shaking or on LB plates at 37°C. 100 µg/ml ampicillin was added to the growth medium for selection.
The transformation of *E. coli* was performed using standard heat-shock protocols.

*Staphylococcus aureus* was grown in brain-heart-infusion (BHI) medium with shaking at 37°C or on BHI agar at 37°C. When appropriate, 5 µg/ml erythromycin was added to the growth medium for selection. Expression from the P_spac promoter was induced by the addition of IPTG to the growth medium. Transformation of *S. aureus* was performed by electroporation, as described before (Lofblom, Kronqvist, Uhlen, Stahl, & Wernerus, 2007), with plasmids isolated from *E. coli* IM08B (Monk, Tree, Howden, Stinear, & Foster, 2015).

### 4.2 | Plasmid and strain construction

All primers and plasmids used in this study are listed in Table 4.

#### 4.2.1 | pLOW-mecA

The mecA gene was amplified from *S. aureus* COL (Gill et al., 2005) using primers mf3 and mf2. The PCR product was digested with restriction enzymes Sall and EcoRI and ligated into the multiple cloning site of pLOW, to produce the plasmid pLOW-mecA, a plasmid with the inducible promoter P_spac that allows controlled expression of mecA. The ligation reaction was transformed into *E. coli* IM08B, and correct constructs were verified by colony PCR and sequencing with primers IM110 and IM134.

#### 4.2.2 | pLOW-mecA(S403A)

The mecA(S403A) allele was made by introducing a point mutation using a two-step overlap extension PCR. The first fragment was amplified with primers mf3 and mecA_S403A_r, and the second fragment was amplified with primers mecA_S403A_f and mf2. pLOW-mecA was used as template DNA. The mutation was introduced by the overlapping inner primers, and the two fragments were fused in a second PCR using the two outer primers which contain the Sall and EcoRI restriction sites. The final fragment was digested with Sall and EcoRI and ligated into the multiple cloning site of pLOW. The resulting plasmid was verified by PCR and sequencing as described above.

| Strain or plasmid | Description | Reference |
|-------------------|-------------|-----------|
| **Escherichia coli** | | |
| IM08B | | Monk et al. (2015) |
| **Staphylococcus aureus** | | |
| RN4220 | | Kreiswirth et al. (1983) |
| COL | | Gill et al. (2005) |
| MK1483 | RN4220, chromosomal integration of SarA_P1-sGFP in the locus between genes SAOUHSC_00038 and SAOUHSC_00039 | This study |
| MF7 | RN4220, pLOW-mecA | This study |
| MF21 | RN4220, pLOW-mecA(S403A) | This study |
| MF23 | RN4220, pLOW-mecA(K188A) | This study |
| MF27 | MK1483, pLOW-mecA(S403A) | This study |
| IM55 | RN4220, pLOW-lacA-gfp | Lab collection |
| **Plasmids** | | |
| pLOW-GFP | Plasmid containing a gfp gene downstream of a P_spac promoter (P_spac-MCS-gfp) | Liew et al. (2011) |
| pLOW-mecA | Expressing mecA from an IPTG-inducible promoter (P_spac-mecA), ery<sup>R</sup>, amp<sup>R</sup> | This study |
| pLOW-mecA(S403A) | Expressing mecA with mutation S403A to inactivate the active site P_spac-mecA(S403A), ery<sup>R</sup>, amp<sup>R</sup> | This study |
| pLOW-mecA(K188A) | Expressing mecA with mutation K188A to inactivate the allosteric site P_spac-mecA(K188A), ery<sup>R</sup>, amp<sup>R</sup> | This study |
| pTH100 | Vector for the integration of SarA_P1-sGFP in the locus between genes SAOUHSC_00038 and SAOUHSC_00039 pJB38-NWMN29-30 + SarA_P1-sGFP-Term | de Jong et al. (2017) |

**TABLE 3** Strains and plasmids used in this study
4.2.3 | pLOW-mecA(K188A)

The mecA(K188A) allele was made by the introduction of a point mutation using a two-step overlap extension PCR. The first fragment was amplified with primers mf3 and mecA_K188A_r, and the second fragment was amplified with primers mecA_K188A_f and mf2. pLOW-mecA was used as template DNA. The plasmid was then made and verified as described above.

4.2.4 | Staphylococcus aureus MK1483

To make a constitutive GFP-positive strain (SarA_P1-sfgfp in RN4220), we used the plasmid pTH100, which allows markerless integration of a superfolder gfp expressing construct in an intergenic region between genes SAOUHSC_00038 and SAOUHSC_00039 (de Jong et al., 2017). The temperature-sensitive pTH100 plasmid was transformed into *S. aureus* RN4220 at 30°C using chloramphenicol as a selection marker, and the double crossover was generated as described (de Jong et al., 2017). GFP-positive colonies were finally verified for correct integration by PCR.

4.3 | Growth assays and determination of β-lactam susceptibility

The MICs for different antibiotics were determined by twofold dilution assays in microtiter plates. Overnight cultures of *S. aureus* strains grown in BHI with 5 µg/ml erythromycin were diluted 100-fold in medium with various IPTG concentrations and exposed to a twofold dilution series of the antibiotics (listed in Table 1). Growth at 37°C was monitored by measuring OD₆₀₀ every 10th minute for 15 hr in a Synergy (BioTek) or Hidex microtiter plate reader (BioTek) with shaking for 5 s before each measurement. MIC (MIC₅₀) was defined as the minimal concentration to inhibit the growth of at least 50%. The fold protection was determined as the ratio between the MIC value for full mecA induction (1,000 µM IPTG) and the MIC value for the uninduced condition. All MIC assays were performed at least three times.

4.4 | Population analysis profile (PAP)

PAPs were performed as described by Reichmann and Pinho (2017) with some modifications. Briefly, overnight cultures of strains MF7 and MF12 with 250 µM IPTG and without IPTG were diluted to 10⁻¹ to 10⁻⁷. Ten µl of each dilution was plated on BHI plates containing cefoxitin (0, 1, 2, 4, 6, and 16 µg/ml), erythromycin 5 µg/ml and 250 mM IPTG when necessary. Plates were incubated at 37°C, and colonies were counted after 24 hr.

4.5 | Time-lapse microfluidics microscopy

A CellASIC® ONIX2 Microfluidic System (Millipore) connected to a Zeiss fluorescence microscope was used to monitor the growth and survival of *S. aureus* during exposure to cefoxitin and cefotaxime. Strains MF7 and MF27 were grown overnight in BHI with 5 µg/ml erythromycin. The cultures were rediluted in the same medium with 250 µM IPTG for induction and grown for 3 hr until the cultures reached the exponential phase (OD₆₀₀ = 0.4). CellASIC® ONIX B04A-03 Microfluidic Bacteria Plates (Millipore) were primed with medium (BHI with 5 µg/ml erythromycin and 250 µM IPTG), and cells were loaded onto the plates according to the manufacturer’s protocol. Images were acquired with a Zeiss Axio Observer with an Orca-Flash4.0 V2 Digital complementary metal-oxide-semiconductor (CMOS) camera (Hamamatsu Photonics) through a 100× PC objective. HPX 120 Illuminator was used as a fluorescent light source. Cells were imaged (phase contrast and GFP fluorescence) every 15th minute for 6 hr during normal growth or exposure to cefoxitin or cefotaxime.

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**CONFLICT OF INTEREST**

None declared.

| Name                      | Sequence (5′-3′)                      |
|---------------------------|--------------------------------------|
| mf3_mecA_f_Sall           | ACTGGTCGACGTAATATACTACAAATGTAGTCTT   |
| mf2_mecA_r_EcoRI          | GATCGAATTCTCGTTACGGATGTTCACTG        |
| im110_seq-pLOW_up_ermC    | TTGTTGATAATGAACGTTGC                 |
| im134_pLOW_down_check_R  | TGTGCTGCAAGGCGATTAAG                 |
| mecA_K188A_f              | AGCAATCGCTgcAGAACTAACTATTTC          |
| mecA_K188A_r              | GAAATCTTTAGTTCTgcAGGCTTTG            |
| mecA_S403A_f              | ACTTCACCAGGTgcAACTCAAAATAT           |
| mecA_S403A_r              | ATATTTTTTGAGTTGacCTGGTGAAGT          |

**TABLE 4** Oligos used in this study
AUTHOR CONTRIBUTION
Marte Ekeland Fergestad: Conceptualization (supporting); investigation (lead); methodology (equal); writing – original draft (supporting); writing – review & editing (lead). Gro Anita Stamssås: Conceptualization (supporting); investigation (equal); methodology (equal); writing – review & editing (equal). Danae Morales Angeles: Investigation (equal); methodology (equal); writing – review & editing (equal). Zhian Salehian: Investigation (equal); methodology (equal); writing – review & editing (equal). Yngvild Wasteson: Conceptualization (supporting); investigation (equal); methodology (equal); writing – review & editing (equal). Morten Kjos: Conceptualization (lead); funding acquisition (equal); investigation (supporting); methodology (equal); supervision (lead); writing – original draft (lead); writing – review & editing (lead).

ETHICS STATEMENT
None required.

DATA AVAILABILITY STATEMENT
Data generated in this study are available from the corresponding author upon request. Movies S1–S5 are available at figshare: https://doi.org/10.6084/m9.figshare.12168351.v1.

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