Received: 25 March 2020 | Revised: 21 April 2020 | Accepted: 25 April 2020

DOI: 10.1002/mbo3.1057

ORIGINAL ARTICLE

MicrobiologyOpen Open Access WILEY

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

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Funding information
Research Council of Norway ANIHWA ERA-Net, Grant/Award Number: 258877; Research Council of Norway FRIMEDBIO, Grant/Award Number: 250976; Research Council of Norway JPIAMR, Grant/Award Number: 296906

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

Marte Ekeland Fergestad and Gro Anita Stamsås contributed equally to this work and are listed with increasing seniority.

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 glycan 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, Banzhaf, 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-ala-D-ala 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 SEDS (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 P_{spac} promoter on a plasmid in the MSSA laboratory strain RN4220 (pLOW-*mecA*, strain MF7). This strain allows 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 (P_{spac}-*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 heterologous 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 (P_{spac}-*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 1b,c). By further increasing the inducer concentration, a maximum of eightfold increase in MIC was obtained compared to the noninduced MF7 strain (Figure 1d,e, Table 1). The MIC of the noninduced MF7-strain was similar to that of the vector control strain (IM55) and the wild-type RN4220, verifying that leakiness of the P_{spac} promoter did not influence the level of resistance (Table 2). We also performed a population analysis profile (Tomasz et al., 1991) of the MF7-strain and control strain toward cefoxitin, by plating the strain onto different concentrations of cefoxitin. The population analysis profile shows a heterogeneous resistant pattern for MF7 (Figure 1g). This result thus suggests that the majority of cells in the population are sensitive to cefoxitin despite expressing *mecA*. This is in line with what has been reported for the heterologous expression of *mecA* before (Katayama, Zhang, Hong, & Chambers, 2003).

To verify that the enzymatic activity of PBP2a is needed for the observed protection, we created a *mecA* mutant construct, in which the active site serine was changed to alanine (S403A). This mutation has previously been shown to abolish the enzymatic activity of PBP2a (Sun, Bauer, & Lu, 1998). As expected, the *mecA*(S403A) mutation (strain MF21) fully abolished the protection by PBP2a in

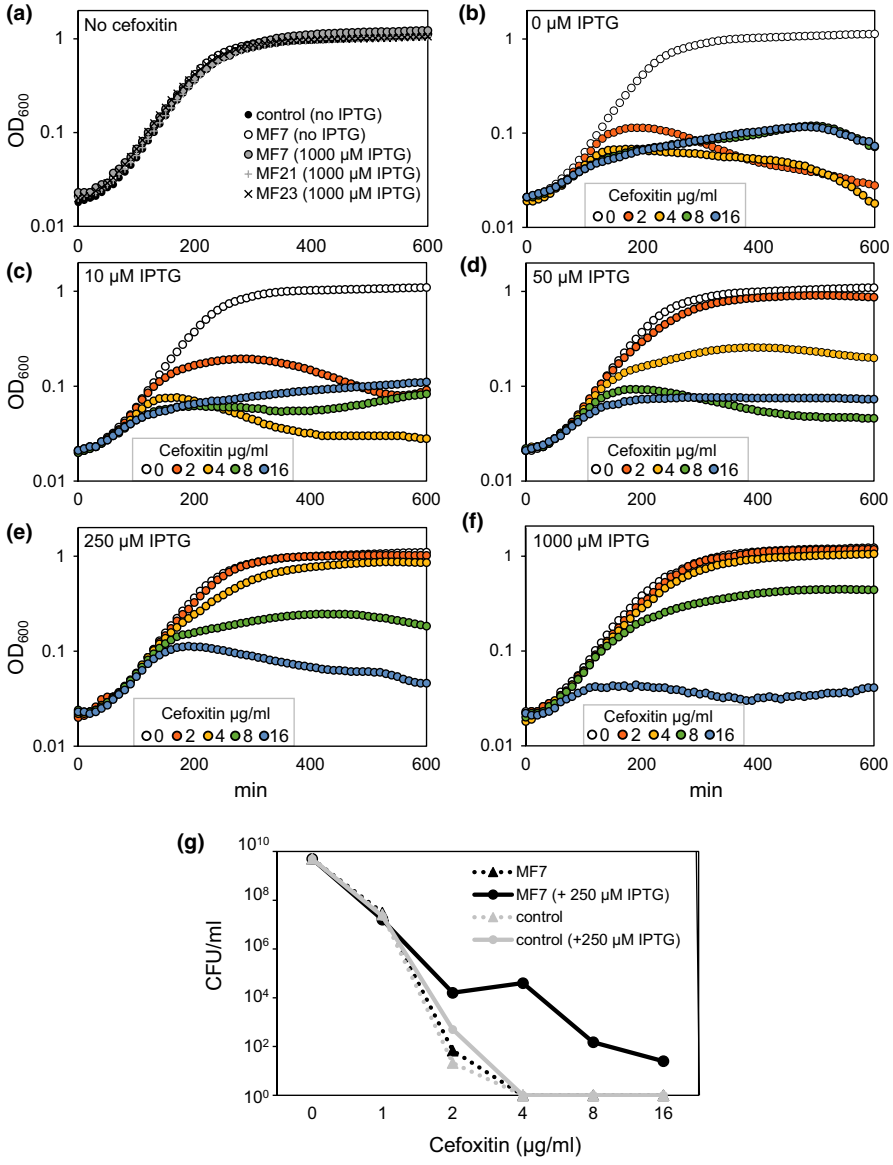

FIGURE 1 Growth of *Staphylococcus aureus* RN4220 expressing *mecA*. (a) Expression of *mecA*, *mecA*(S403A) or *mecA*(K188A) in *S. aureus* does not affect growth. Growth of MF7 (P_{spac}-*mecA*), MF21 (P_{spac}-*mecA*(S403A)), and MF23 (P_{spac}-*mecA*(K188A)) with maximum *mecA* induction (1,000 μM 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 (P_{spac}-*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

TABLE 1 Level of protection by *mecA* expression in *Staphylococcus aureus* MF7 for different β-lactams[a]

β-lactam[b]	Class	MIC RN4220 (μg/ml)	MIC MF7 (μg/ml)		Fold protection[c]
			No IPTG	1,000 μM IPTG	
Ampicillin	3rd generation penicillin (extended spectrum)	0.78	0.78	12.5	16
Cefaclor	2nd generation cephalosporin	0.5	0.5	16	32
Cefotaxime	3rd generation cephalosporin	0.5	0.5	4	8
Cefoxitin	2nd generation cephalosporin	1	1	8	8
Cephalexin	1st generation cephalosporin	2	2	64	32
Imipenem	Carbapenem	0.03	0.03	0.13	4
Oxacillin	2nd generation penicillin (narrow spectrum)	0.39	0.39	6.25	16
Penicillin G	1st generation penicillin (narrow spectrum)	0.10	0.10	0.78	8
Piperacillin	4th generation penicillin (extended spectrum)	1.56	1.56	12.5	8

[a]The experiments were repeated at least three times with similar results.

[b]Among the β-lactams tested here, four have been reported to have a specific affinity for certain PBPs in *S. aureus*. These are cefaclor (specific to PBP3), cefotaxime (PBP2), cefoxitin (PBP4), and cephalexin (PBP3).

[c]Fold protection by *mecA* induction was determined as the ratio between MIC with induction and the MIC of uninduced cells.

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_{spac}-*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, Matsuhashi, & 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 cephalexin 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 PBP1-PBP4 (Chambers & Sachdeva, 1990; Georgopapadakou et al., 1982, 1983, 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 cephalexin 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 cephalexin. 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[a]

ABX	Strain	Genotype	Concentration IPTG (μM)				Fold protection[b]
			0	50	250	1,000	
Cefoxitin	IM55	Control	1	1	1	1	1
	MF7	P_{spac}-*mecA*	1	2	4	8	8
	MF21	P_{spac}-*mecA* (S403A)	1	1	1	1	1
	MF23	P_{spac}-*mecA* (K188A)	1	2	8	8	8
Cefotaxime	IM55	Control	0.5	0.5	0.5	0.5	1
	MF7	P_{spac}-*mecA*	0.5	2	4	4	8
	MF21	P_{spac}-*mecA* (S403A)	0.5	0.5	0.5	0.5	1
	MF23	P_{spac}-*mecA* (K188A)	0.5	1	4	4	8
Cefaclor	IM55	Control	0.5	0.5	0.5	0.5	1
	MF7	P_{spac}-*mecA*	0.5	4	16	16	32
	MF21	P_{spac}-*mecA* (S403A)	0.5	0.5	0.5	0.5	1
	MF23	P_{spac}-*mecA* (K188A)	1	4	16	16	32
Cephalexin	IM55	Control	2	2	2	2	1
	MF7	P_{spac}-*mecA*	2	8	64	64	32
	MF21	P_{spac}-*mecA* (S403A)	2	2	2	2	1
	MF23	P_{spac}-*mecA* (K188A)	2	16	32	64	32

[a]The experiments were repeated at least three times with similar results.

[b]Fold 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

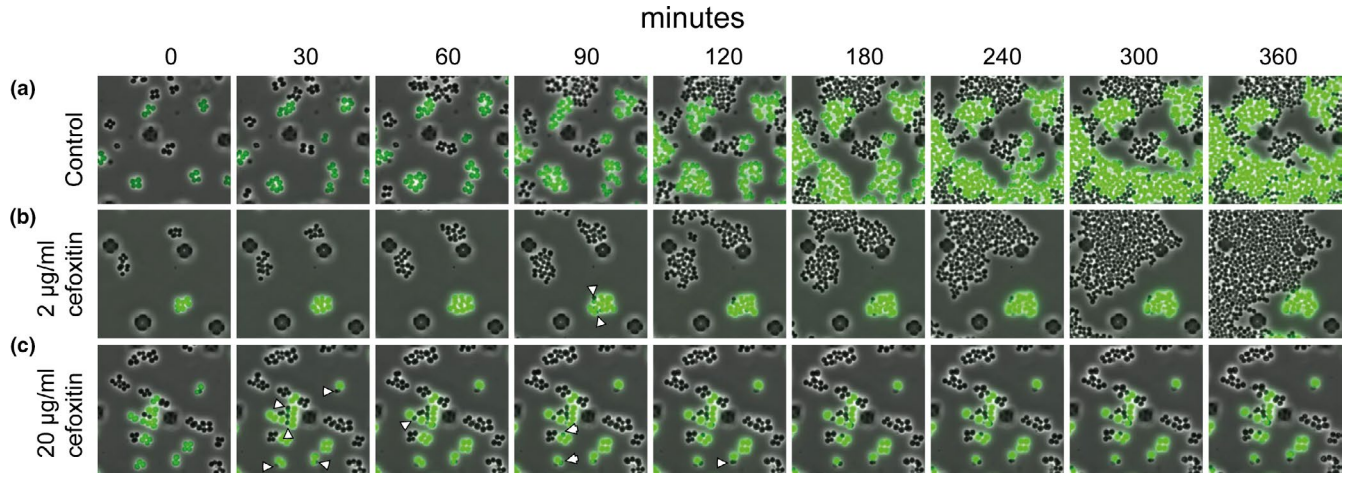

FIGURE 2 Microfluidics fluorescence microscopy time-lapse experiments in the presence of different concentrations of cefoxitin. The two strains MF7 (P_{spac}-*mecA*, dark cells) and MF27 (P_{spac}-*mecA*(S403A)), GFP+ green cells) were mixed in equal ratios, and pregrown in media with 250 μ IPTG to induce 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)

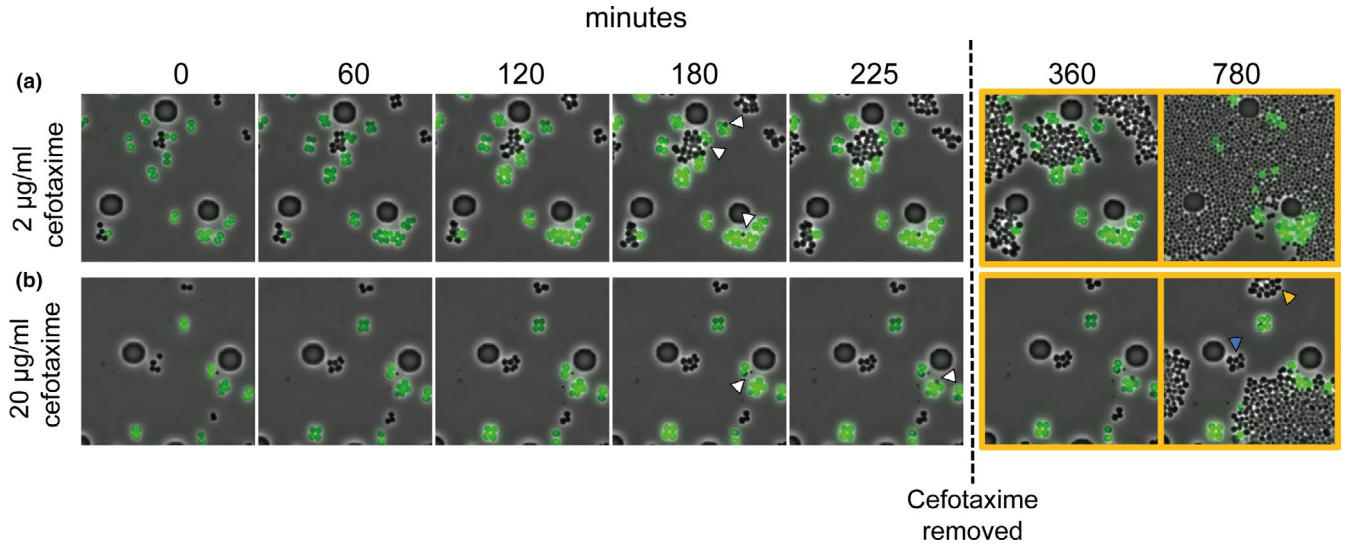

FIGURE 3 Microfluidics fluorescence microscopy time-lapse experiments in the presence of different concentrations of cefotaxime. The two strains MF7 (P_{spac}-*mecA*, dark cells) and MF27 (P_{spac}-*mecA*(S403A)), GFP+ green cells) were mixed in equal ratios, and pregrown in media with 250 µM IPTG to induce expression of the *mecA* alleles. Single-cell growth was analyzed in medium (a) with 2 µg/ml and (c) 20 µg/ml cefotaxime using a CellASIC ONIX Microfluidics setup. 250 µM IPTG was present in all conditions to induce the expression of *mecA* alleles. After 225 min, the cefotaxime-containing medium was changed to regular medium to investigate whether any of the cells could recover. White arrowheads point to lysing cells. Yellow and blue arrowheads point to examples of cell regrowing and not regrowing, respectively, after removal of cefotaxime from the media. See also Movies S4–S5 (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 SalI 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 SalI and EcoRI restriction sites. The final fragment was digested with SalI and EcoRI and ligated into the multiple cloning site of pLOW. The resulting plasmid was verified by PCR and sequencing as described above.

TABLE 3 Strains and plasmids used in this study

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-*sfgfp* 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^R, amp^R	This study
pLOW-mecA(S403A)	Expressing *mecA* with mutation S403A to inactivate the active site P_{spac}-*mecA(S403A)*, ery^R, amp^R	This study
pLOW-mecA(K188A)	Expressing *mecA* with mutation K188A to inactivate the allosteric site P_{spac}-*mecA(K188A)*, ery^R, amp^R	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 4 Oligos used in this study

Name	Sequence (5′–3′)
mf3_mecA_f_SalI	ACTGGTCGACGTAATATACTACAAATGTAGTCTT
mf2_mecA_r_EcoRI	GATCGAATTCTCGTTACGGATTGCTTCACTG
im110_seq-pLOW_up ermC	TTGGTTGATAATGAACTGTGCT
im134_pLOW_down_check_R	TGTGCTGCAAGGCGATTAAG
mecA_K188A_f	AGCAATCGCTgcAGAACTAAGTATTTC
mecA_K188A_r	GAAATACTTAGTTCTgcAGCGATTGCT
mecA_S403A_f	ACTTCACCAGGTgCAACTCAAAAAATAT
mecA_S403A_r	ATATTTTTTGAGTTGcACCTGGTGAAGT

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 OD_{600} 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 (MIC_{50}) 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 (OD_{600} = 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.

ACKNOWLEDGMENTS

We would like to thank Ine Myrbråten, NMBU, for help with strain construction. This work has been funded by the ANIHWA ERA-Net project MRSA_BACTERIOPHAGE project (project number 258877), by a FRIMEDBIO grant (project number 250976) and a JPIAMR grant (project number 296906) from the Research Council of Norway.

CONFLICT OF INTEREST

None declared.

AUTHOR CONTRIBUTION

Marte Ekeland Fergestad: Conceptualization (supporting); investigation (lead); methodology (equal); writing – original draft (supporting); writing – review & editing (lead). **Gro Anita Stamsås:** 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); funding acquisition (equal); supervision (supporting); writing – original draft (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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How to cite this article: Fergestad ME, Stamsås GA, Morales Angeles D, Salehian Z, Wasteson Y, Kjos M. Penicillin-binding protein PBP2a provides variable levels of protection toward different β-lactams in *Staphylococcus aureus* RN4220. *MicrobiologyOpen*. 2020;9:e1057. https://doi.org/10.1002/mbo3.1057