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MprF-mediated daptomycin resistance

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ABSTRACT

Daptomycin has become an important antibiotic for the treatment of serious Methicillin-Resistant *Staphylococcus aureus* (MRSA) infections. Unlike other approved antibiotics, its mode of action is still under active investigation, as well as the molecular basis of daptomycin resistance, which emerges in some cases during daptomycin treatment. Small nucleotide polymorphisms (SNPs) in the Multiple Peptide Resistance Factor (MprF) appear to play a major role in the resistance mechanism. Until recently, the impact of the SNPs on MprF activity has remained unclear, which is due to conflicting reports on resistance-associated phenotypes and an incomplete understanding of the mode of action of MprF. However, recent structural insights into MprF and studies with isogenic mutants have now led to a new model of MprF-mediated daptomycin resistance, which harmonizes most of the observed phenotypes and provides a basis for challenging biochemical investigations.

1. Daptomycin and daptomycin resistance

The lipopeptide antibiotic daptomycin has become an important antibiotic for the treatment of serious Methicillin-Resistant *Staphylococcus aureus* (MRSA) infections (Seaton et al., 2016). While some basic properties of its bactericidal mode of action were elucidated (Bayer et al., 2013; Muller et al., 2016; Straus and Hancock, 2006), it is still unclear how daptomycin is able to kill *S. aureus* cells. In the model organism *Bacillus subtilis*, daptomycin was shown to associate with fluid membrane microdomains, which leads to oligomerization and subsequent translocation of daptomycin from the outer to the inner membrane leaflet (Fig. 1). The presence of daptomycin in fluid membrane microdomains was shown to prevent the association of essential membrane proteins with fluid microdomains, resulting in cell death (Muller et al., 2016). To this date the exact target of daptomycin remains unknown (Schneider et al., 2009). The approach of raising spontanously antibiotic-resistant mutants is often used for target identification. Such studies as well as the characterization of daptomycin-resistant (DAP-R) clinical isolates, which had emerged in patients during daptomycin therapy, consistently identified specific single nucleotide polymorphisms (SNPs) in the Multiple Peptide Resistance Factor (MprF) protein (Bayer et al., 2013). However, MprF is not essential for the viability of *S. aureus* and its inactivation leads to daptomycin hypersusceptibility (Ernst et al., 2009; Peschel et al., 2001), indicating that it is not the target of daptomycin but rather protects the bacterial cell from daptomycin in the presence of specific, DAP-R-conferring mutations. The role of MprF in the virulence of *S. aureus* is well-established. MprF confers broad-spectrum resistance to cationic antimicrobial peptides (CAMPs), such as defense peptides produced by the human host or bacteriocins produced by competing microorganisms, thereby enabling *S. aureus* to colonize and infect the human host (Peschel et al., 2001). Daptomycin is an antimicrobial peptide, which shares many properties with CAMPs upon calcium binding (Straus and Hancock, 2006).

2. Basic functions of non-mutated MprF

When MprF was discovered in 2001, it was shown to produce the cationic phospholipid lysylphosphatidyl-glycerol (LysPG), which decreases the negative cell surface charge, leading to the electrostatic repulsion of CAMPs (Peschel et al., 2001). Furthermore, it was shown to be a crucial virulence factor and to be widespread in Gram-positive and Gram-negative bacteria. The substrates for LysPG biosynthesis had already been discovered in 1966 by Lennarz et al (without knowing MprF): LysPG production was shown to depend on the transfer of lysine residues from Lys-tRNA to the negatively charged phospholipid phosphatidylglycerol (PG) (Lennarz et al., 1966). MprF consists of a large hydrophobic N-terminus and a hydrophilic C-terminus (˜300 amino

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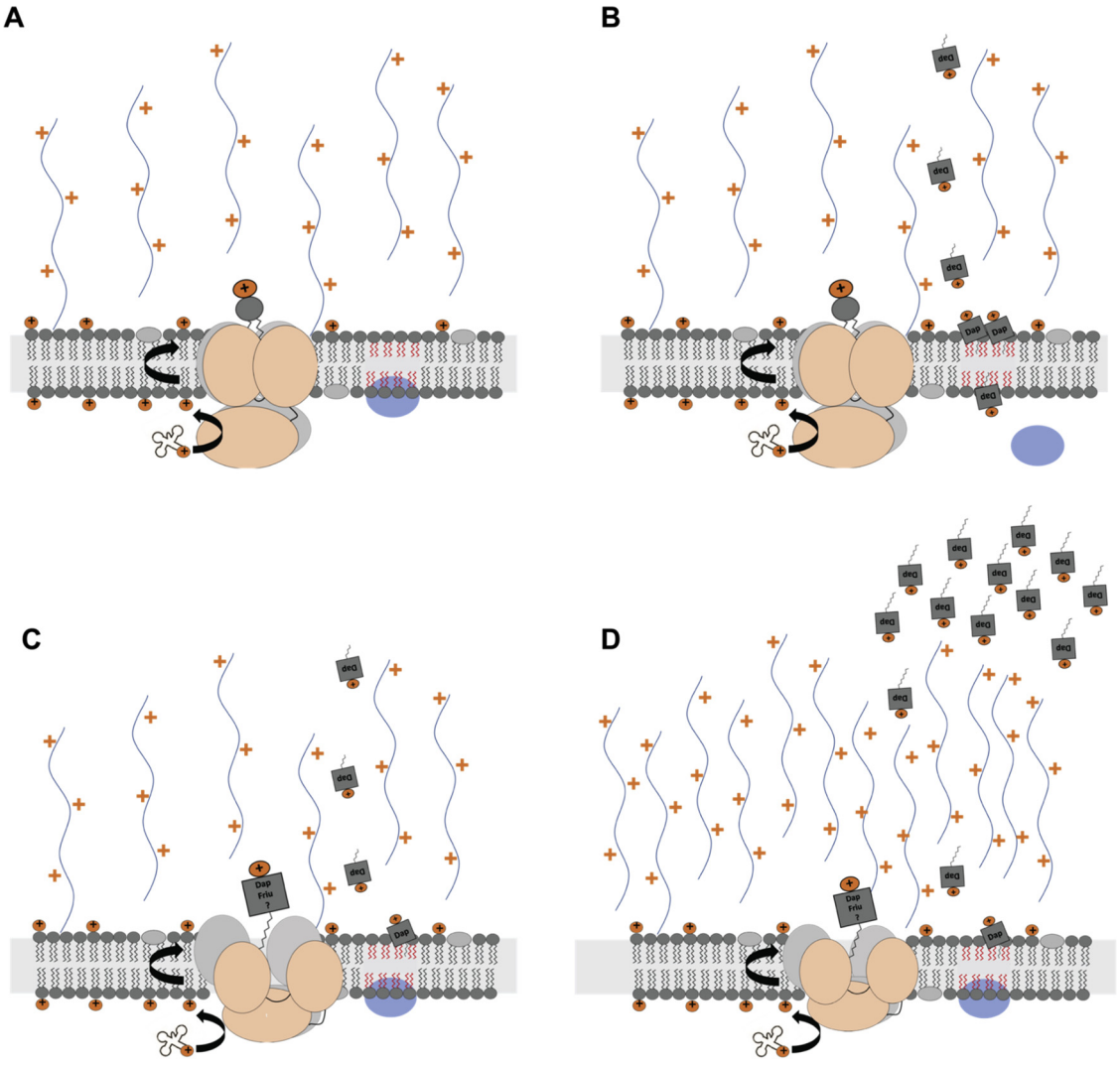

Fig. 1. Proposed mechanism of MprF-mediated DAP-R. A, Oligomerized wild-type MprF (depicted as dimer) synthesizes the cationic phospholipid LysPG by attaching lysine residues from Lys-tRNA to phosphatidylglycerol, and flips LysPG from the inner to the outer membrane leaflet. The presence of positively charged LysPG in the outer membrane leaflet, together with the D-alanylation of negatively charged teichoic acids (indicated) results in repulsion of CAMPs and cationic antibiotics. Essential membrane proteins (blue) associate with fluid lipid microdomains (shown as short phospholipids). B, Daptomycin is attracted to negatively charged phospholipids and associates with fluid microdomains, which leads to oligomerization and subsequent translocation of daptomycin from the outer to the inner membrane leaflet. The presence of daptomycin in fluid microdomains prevents the association of essential membrane proteins with fluid microdomains, resulting in cell death (Muller et al., 2016). C, The point mutation T345A reduces MprF intramolecular interactions, which may enable the flippase to accommodate daptomycin and remove it from fluid microdomains. Alternatively, the flippase may interact with an unknown membrane-embedded molecule that is crucial for the activity of daptomycin, thereby preventing the oligomerization and subsequent translocation of daptomycin. D, DAP-R signature point mutations in MprF, which cannot confer resistance on their own (e.g. S295 L), may have a weaker impact on MprF intramolecular interactions, resulting in less efficient interactions with daptomycin or a molecule that is essential for daptomycin activity. Additional mutations leading to increased teichoic acid production and increased D-alanylation of teichoic acids may reduce the interaction of daptomycin with the membrane, and thereby enable less effective DAP-R signature point mutations in MprF to confer DAP-R (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

acids). Shortly after the identification of mutations in the *mprF* of DAP-R isolates, we found that the hydrophilic C-terminus mediates LysPG synthesis and that MprF actually has a second function that is accomplished by the hydrophobic domain: the flipping of LysPG from the inner to the outer leaflet of the cytoplasmic membrane (Ernst et al., 2009). This process was found to be essential for the electrostatic repulsion of CAMPs and for providing a basic level of protection against daptomycin without reaching a degree of insensitivity that would render bacteria resistant to daptomycin therapy.

3. DAP-R associated point mutations in MprF

Based on knowledge on enzymatic activities of the two parts of MprF, the gain of function of mutated MprF was suspected to increase either the LysPG synthase activity, the flippase activity, or both, as all these changes would lead to an increased presence of LysPG in the outer membrane leaflet, which could potentially increase the electrostatic repulsion of CAMPs to a level that may confer DAP-R (Bayer et al., 2013; Ernst and Peschel, 2011). However, an overall increase of LysPG in the outer leaflet of the membrane was not consistently observed, with some DAP-R isolates not displaying any change in MprF activity at all and some isolates displaying increased LysPG production without an increase in LysPG flipping, which cannot be explained by different types of point mutations in *mprF* (Bayer et al., 2015; Mishra and Bayer, 2013; Rubio et al., 2012). Moreover, other seemingly MprF-unrelated changes were frequently observed in clinical DAP-R isolates, such as increased cell wall production, including the increased neutralization of charged teichoic acids with D-alanine (Bertsche et al., 2011, 2013; Mishra et al.,

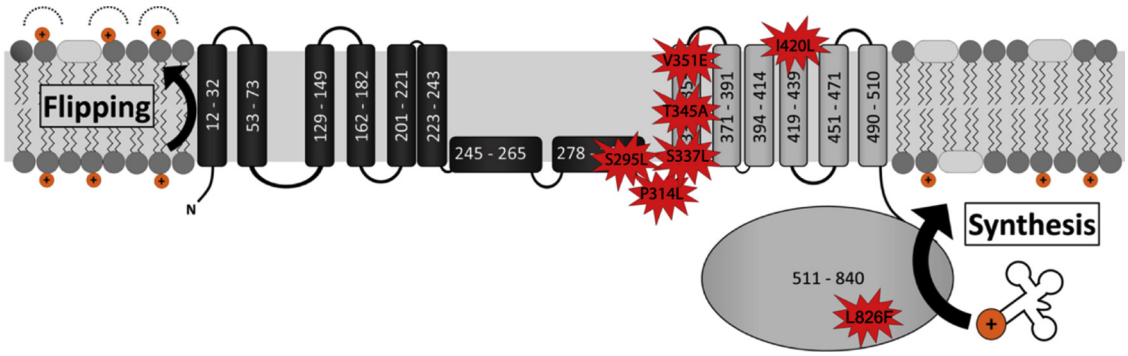

Fig. 2. Topology of MprF and position of the most frequent DAP-R-associated point mutations in MprF. The MprF synthase and flippase domains are shown in gray and black, respectively (Ernst et al., 2018).

2014), which was also shown to be another crucial mechanism of *S. aureus* to resist CAMPs (Peschel and Sahl, 2006). An overall decrease in the negative cell surface charge together with cross-resistance to various cationic antimicrobials was observed in some DAP-R mutants (but not consistently) (Bayer et al., 2015, 2016), which could theoretically be due to increased presence of LysPG in the outer leaflet of the membrane, increased D-alanylation of teichoic acids, or both. Importantly, even though it might be tempting to draw this conclusion, it is possible that none of the observed changes are responsible for the observed DAP-R.

The contribution of characteristic SNPs in MprF to DAP-R was established early with passaging experiments in which mutations in MprF were found to be the first to emerge and to confer two to threefold decreased daptomycin susceptibility (Friedman et al., 2006). In order to systematically study the role of such signature DAP-R mutations on MprF activity in a defined strain background, we analyzed the impact the mutations in the well-characterized laboratory strain SA113 (Ernst et al., 2018). Several signature point mutations (S295 L, P314 L, S337 L, T345A, V351E, I420 N, L826 F) were engineered into *mprF* and cloned in the SA113 *mprF* deletion mutant (Fig. 2). Only two mutations were able to confer DAP-R (T345A and V351E) and, surprisingly, they did not affect LysPG production or translocation. We also did not detect any differences in D-alanylation of teichoic acids or in cell surface charge in these defined mutants, suggesting that the SNPs were not affecting other processes that would result in increased electrostatic repulsion of CAMPs, including daptomycin. Consequently, we also did not detect broad-range cross-resistance to other CAMPs.

A similar study was also conducted in the *S. aureus* Newman background with plasmid-based expression of *mprF* containing the DAP-R-associated signature mutations S295 L and T345A in *a ΔmprF* mutant (Yang et al., 2013). In the Newman strain background, both mutations were shown to increase the daptomycin MIC substantially (four and eightfold, respectively, compared to corresponding un-mutated *mprF*). In contrast, our study reported that only the T345A mutation conferred DAP-R, without affecting synthesis or translocation of LysPG into the outer membrane leaflet (Ernst et al., 2018). Conflicting findings were reported by Yang et al. (Yang et al., 2013), which found that both mutations led to overall increased LysPG production and decrease in negative cell surface charge when expressed in Newman *ΔmprF*. However, the LysPG and surface charge levels did not exceed those observed in the (daptomycin-susceptible) *S. aureus* Newman wild type, suggesting that these phenotypes were not contributing to DAP-R. Finally, a third study investigated the impact of the signature mutation L826 F in the background of clinical isolates (Mehta et al., 2012). Here, in contrast to our study, L826 F was found to confer DAP-R, but to have no impact on the cell surface charge, again suggesting that increased LysPG accumulation in the outer membrane leaflet was not involved in the resistance mechanism. Taken together, these three studies with isogenic mutants demonstrate the causal role of MprF mutations in DAP-R in different strain backgrounds and show that resistance cannot be explained by the known enzymatic activities of MprF. The observed conflicting phenotypes appear to be due to additional factors, which illustrates the difficulties of unveiling this critical antibiotic resistance mechanism.

4. Model for the mechanism of MprF-mediated DAP-R

The above-described studies with isogenic mutants, together with the phenotypes observed in clinical DAP-R isolates, suggested that DAP-R is not mediated by electrostatic repulsion and that additional processes contribute to DAP-R. In fact, the role of additional factors was established in the first reported passaging experiments, in which, shortly after the emergence of mutations in *mprF,* additional point mutations in other loci emerged, which further decreased daptomycin susceptibility (Friedman et al., 2006). Thus, the multiple, often conflicting phenotypes observed in clinical isolates may be the result of additional mutations, already existing prior to or acquired during daptomycin treatment, which may explain why some of the mutations did not confer resistance in the SA113 strain background on their own (Ernst et al., 2018). On the other hand, additional mutations may in some cases affect cell-envelope related phenotypes that may not necessarily contribute to DAP-R, thereby complicating investigations aimed at elucidating the molecular mechanism of DAP-R.

How then is mutated MprF contributing to DAP-R if none of the known enzymatic activities of MprF is affected by the point mutations? One important piece of the puzzle was our finding that a DAP-R conferring SNP (T345A) caused cross-resistance only to the structurally related lipopeptide antibiotic friulimicin B (Ernst et al., 2018), which has a different target than MprF (Schneider et al., 2009). This suggested that the DAP-R mechanism is rather specific for the structure of daptomycin-related antibiotics and is probably not directly related to interaction with its target. The other important piece of the puzzle emerged from the thorough structural investigation of MprF, in which we determined the topology and mapped the flippase to a distinct N-terminal domain in the hydrophobic part of MprF (Ernst et al., 2015). This showed that none of the signature DAP-R point mutations in *mprF* were located in the flippase domain but were located right next to it at the junction of the flippase and the synthase domain, with exception of the L826 F mutation, which is located in the cytosolic part of the synthase domain (Fig. 2). The accumulation of DAP-R conferring mutations outside of the flippase domain suggested that the function of the flippase domain was essential for the DAP-R resistance mechanism. Indeed, we found that DAP-R could not be conferred when essential sites of the flippase were inactivated (Ernst et al., 2018). Importantly, we found that MprF is actually an oligomer in which different domains of MprF interact distinctively with each other, which suggested that the oligomerization of MprF was creating a translocation channel facilitating LysPG flipping (Ernst et al., 2015). The DAP-R conferring T345A

mutation had no impact on overall oligomerization (Ernst et al., 2018), but it did lead to reduced intramolecular interactions between the flippase and the synthase domain, which is likely to affect the structure of the translocation channel.

To harmonize all these observations we suggested that the signature DAP-R mutations in *mprF* might further extend the substrate specificity of the flippase (Fig. 1), which was previously found to also accommodate alanyl-phosphatidylglycerol (AlaPG) in addition to Lys-PG (Slavetinsky et al., 2012). Thus, the DAP-R-conferrring SNPs might enable the translocation of daptomycin itself or of a so far unidentified membrane-embedded molecule that is important for the activity of daptomycin and friulimicin B (Ernst et al., 2018) (Fig. 1C). Our model of MprF-mediated DAP-R could also explain how the L826 F mutation in the cytosolic LysPG synthase domain can confer resistance in certain strain backgrounds, as the cytosolic part was found to be involved in multiple inter-domain interactions (Ernst et al., 2015), including interaction with itself, and therefore might have a similar effect on the translocation channel as the point mutations at the junction between LysPG synthase and flippase (Fig. 2).

All of these findings are supportive for a model in which all of the signature DAP-R mutations in *mprF* enable the translocation of daptomycin or another membrane-embedded molecule that is crucial for daptomycin activity, which might prevent the accumulation of daptomycin in fluid microdomains (Fig. 1). Some mutations (T345A, V351E) appear to be more effective in extending the substrate specificity of the flippase than others (Ernst et al., 2018), which may require additional mutations in other proteins that can for instance reduce the diffusion of daptomycin across the cell wall to the membrane. Based on the current knowledge and findings on DAP-R in clinical isolates, the most common additional factor is likely to be the increased D-alanylation of teichoic acids (Bertsche et al., 2011, 2013; Mishra et al., 2014), which may occur during daptomycin treatment or may have already occurred prior to daptomycin treatment (Wanner et al., 2017). Changes in teichoic acid production and D-alanylation may not only enable DAP-R in the background of mutations with weaker capacities to confer DAP-R in *mprF* than T345A, but may further increase the resistance level of DAP-R isolates (Fig. 1D). Of note, it is possible that the proposed extended substrate specificity of the flippase may lead to some distinct changes in the membrane proteome (Sievers et al., 2010), which may affect other cellular processes and thereby contribute to additional cell-envelope related phenotypes that are seen in DAP-R isolates, such as decreased membrane fluidity (Mishra et al., 2009), increased cell wall thickening (Yang et al., 2010), and the frequently observed increased susceptibility to β-lactam antibiotics (so called 'see-saw effect') (Renzoni et al., 2017).

5. Future perspectives

In conclusion, studies on DAP-R have been complicated by multiple additional phenotypic changes in clinical isolates, often resulting in surface charge-related phenotypes that also appeared to have the potential to confer DAP-R, in addition to the specifically DAP-R-conferring mutations in MprF. It is not entirely clear what these additional mutations are, since whole genome sequencing was only rarely employed to systematically investigate DAP-R and this approach is not able to detect changes that had already occurred in parental isolates. Future, prospective whole-genome sequencing based studies should help to create a more comprehensive picture of the processes leading or contributing to DAP-R. Based on the current knowledge it appears that cell wall stress regulons are often dysregulated in DAP-R mutants (Bayer et al., 2013). However, recent investigations with isogenic isolates demonstrated the key role of mutations at the junction of the LysPG synthase and flippase of MprF in DAP-R, which together with recent fundamental investigations on the mode of action of MprF have for the first time led to a plausible model for the resistance mechanism.

It will be challenging to biochemically verify the proposed extended substrate specificity of the MprF flippase domain caused by the point mutations in reconstituted membranes with purified MprF proteins, not only because of the difficulties of purifying extremely hydrophobic proteins but also because of the dynamics of the flipping process, which may not occur unidirectionally. Yet another challenge will be to conduct structural investigations of the translocation channel with MprF crystals, which will be a difficult undertaking due to the size and hydrophobicity of the protein. Insights from such studies will not only lead to a complete understanding of the DAP-R mechanism but also to fundamental insights into phospholipid translocation, as MprF is the first documented bacterial phospholipid flippase.

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