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Genetic basis of methicillin resistance in *Staphylococcus aureus*

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Abstract. Methicillin resistance in staphylococci is due to the acquisition of the *mecA* gene encoding a new penicillin-binding protein (PBP2′, PBP2a) that has a lower affinity to methicillin than the endogenous PBPs. PBP2′ is involved in the assembly of the cell wall peptidoglycan in the presence of high concentrations of β-lactams that otherwise inhibit the endogenous PBPs. The production of PBP2′ is under dual control by its own *mecR1-mecI*- and the penicillinase *blaR1-blaI*-encoded regulatory elements. Resistance to high levels of methicillin depends, in addition to PBP2′, on chromosomally encoded factors that are involved in the synthesis and degradation of the peptidoglycan. Any mutations that reduce peptidoglycan precursor formation or change the chemical composition of the muropeptide precursor result in lowered resistance.

Key words. *Staphylococcus aureus*; methicillin resistance; *mecA*; *fem*; penicillin-binding protein; PBP2a; PBP2′; peptidoglycan.

Introduction

The first targets of β-lactams in *Staphylococcus aureus* are the four penicillin-binding proteins (PBPs) [1, 2]. The interaction of β-lactams with the PBPs takes place on the outer surface of the cytoplasmic membrane and leads to a reduction in peptidoglycan cross-linking and loss of the splitting system. The time course and details of β-lactam-induced morphogenetic changes and death in *S. aureus* have recently been re-evaluated in an excellent review [3]. Staphylococci have developed, in addition to, penicillinases, a further powerful resistance mechanism against virtually all β-lactam antibiotics and their derivatives, namely methicillin resistance. Methicillin-resistant *S. aureus* (MRSA) have the tendency to accumulate additional resistance determinants, resulting in the formation of multiply resistant MRSA, which are creating increasing therapeutic problems. This development peaked recently with the isolation of vancomycin-resistant MRSA [4] which threaten the efficacy of the last antibiotic used for therapy of multiply resistant MRSA. There is therefore an imminent need to identify novel targets for new antibacterial agents and to devise new strategies to control resistance.

Biology and evolution of the methicillin resistance determinant

The first methicillin-resistant clones of *S. aureus* appeared in 1961 when methicillin, the first penicillinase-resistant penicillin, was introduced into clinical use [5]. Since then, methicillin resistance seems to have emerged on various occasions, and in different clonal lineages of *S. aureus* [6]. Methicillin resistance is due to the acquisition of the *mecA* gene which codes for a penicillin-binding protein PBP2′ (PBP2a) [7]. The 2-kb *mecA* gene is localised on a 32- to over 60-kb element, the *mec* element, that has no allelic equivalent in susceptible strains. It carries attachment sites for transposons and at least one IS*257* (IS*431*$_{mec}$) sequence which acts as a trap for the capture of further IS*257*-linked resistance determinants and resistance plasmids. This leads to a clustering of multiple resistance determinants in that part of the DNA [8]. The structure and behaviour of the *mec* element suggest it to be an unusual site-specific transposon: it carries sequences for at least two recombinases, it is framed by inverted repeats, and integrates into the chromosome without generating direct repeats [9]. Its integration is site- and orientation-specific in relation to *gyrA* [10]. The sequence of the *mecA* gene is

well preserved between different MRSA and methicillin-resistant coagulase-negative strains [11]. Genetic and epidemiological studies suggest that the *mec* element is acquired in *S. aureus* by horizontal transfer from methicillin-resistant coagulase-negative staphylococci [12]. The mode of strain-to-strain transfer has, however, not yet been elucidated. The origin of the *mec* element is still unclear. A *mecA*-like gene with $>80\%$ sequence homology to the MRSA *mecA* is found in all strains of the animal species *Staphylococcus sciuri* [13], leading to the hypothesis that the *S. sciuri mecA* gene may be an evolutionary relative of the PBP2′-encoding *S. aureus mecA*, although the native *S. sciuri mecA* gene does not confer methicillin resistance [14].

The *mec* operon and its regulation

The *mec* element has entered, on various occasions, different *S. aureus* strains creating distinct lineages of MRSA that vary in their genetic background. Three types of *mec* elements that differ in size and genetic components have been described [9]. Their common core comprises the *mecA* gene and the divergently transcribed regulatory elements *mecR1-mecI* [15]. MecR1 has sequence homology to the *Bacillus licheniformis* and the staphylococcal penicillin sensory transducer BlaR1 [16, 17]. It is a transmembrane β-lactam-sensing protein that acts as a signal transducer in β-lactam induction of *mecA* [18] and carries a cytoplasmic-oriented Zn-peptidase motif. MecI acts as a repressor of *mecA* [19] and shows homology to the β-lactamase repressor BlaI. In some clinical isolates, the sequences comprising *mecR1-mecI* are partially deleted and replaced by a truncated IS*1272* [20] or have mutations in *mecI* or the promoter region [21–23] leading to constitutive PBP2′ production. The *mecA* gene is under dual control [24, 25]. BlaI, the repressor of the β-lactamase, can also bind to the operator region of *mecA*. Upon induction by β-lactams, the BlaI repressor is proteolytically cleaved [24] and *mecA* transcription resumes. Repression of *mecA* by MecI is stronger than by BlaI [25], although interactions of BlaI with synthetic *mec* and *bla* operator sequences were shown to be equally strong [24]. Regulation of *mecA* transcription seems to depend strongly on the cross-talk between inducer (β-lactam)/signal-transducer (BlaR1 and/or MecR1), and signal transmission to the repressor. Further elements in the β-lactam induction cascade are postulated, such as the chromosomally encoded BlaR2 which is required for plasmid-mediated β-lactamase expression in *S. aureus* [26]. The close relationship between the *mec* and *bla* regulatory elements indicates that BlaR2 or a close relative may also be involved in *mecA* induction.

Resistance levels in relation to PBP2′ and β-lactamase production

The transmembrane signal-transducing BlaR1 responds to virtually all β-lactams [27]. MecR1, on the contrary, is selective and does not react to oxacillin or methicillin but to cefoxitin [28]. MRSA strains with an intact regulatory element may therefore appear phenotypically susceptible to methicillin despite the presence of *mecA*. Introduction of a multicopy plasmid containing the regulatory elements *mecR1-mecI* into an MRSA with constitutive *mecA* transcription reduces both *mecA* transcription and methicillin resistance [29], presumably due in part to the copy number effect. However, one MRSA tested was not affected in resistance upon inactivation of *mecI* or overproduction of *mecR1-mecI*, showing that the effect of fully functional *mecR1-mecI* and intact operator sequences on *mecA* transcription or resistance levels may depend upon the strain background [29]. Likewise, introduction of a penicillinase plasmid into the MRSA strain COL rendered PBP2′ synthesis inducible but did not affect methicillin resistance levels, and only after inactivation of BlaR1 was resistance reduced [27]. This indicates that there may be further elements, e.g. *blaR2*, involved in induction. In *S. epidermidis*, there is an interesting relationship between lack of *mecA* transcription in slime-negative phase variants [30]; there, *mecA* is thought to be under the control of an accessory gene-regulator-like regulatory network [31].

The lack of correlation between resistance levels and amount of PBP2′ production lead to the conclusion that genes other than *mecA*, *mecR1-mecI* and *blaR1-blaI* are responsible for the strain-specific differences in resistance.

Heteroresistance in MRSA

An intriguing feature of methicillin resistance is the phenomenon referred to as heteroresistance. Heterogeneous MRSA consist of different subpopulations, including a small minority (10^{-8}–10^{-4}) of cells with resistance to high concentrations of methicillin, and a majority of cells with a resistance sometimes just above that of susceptible strains [32]. The number of highly resistant subpopulations and their frequency within an MRSA is a strain-specific, reproducible property. Usually, with few exceptions, the highly resistant subclones retain their high resistance once they have been isolated. The same mechanism is thought to operate in the clinical environment, leading to the selection of strains with increasing resistance, one of the reasons for the failure of β-lactam treatment against MRSA. MRSA strains were divided into four arbitrary expres-

sion classes according to their resistance profiles [33], ranging from highly homogeneously resistant strains to strains with various degrees of heterogeneous resistance. There is, however, as yet no mechanistic or genetic model available that correlates with these different expression classes. Genetic manipulations showed that *mecA* is the primary genetic determinant of methicillin resistance, but it requires the function of additional chromosomal genes for optimal resistance [34, 35]. Besides the genomic differences that affect resistance levels, methicillin resistance in MRSA is strongly dependent upon the growth medium [36] and other external factors like temperature, pH, osmolarity, divalent cations, and anaerobiosis [reviewed in ref. 37].

Chromosomal genes affecting methicillin resistance levels

Methicillin resistance can be reduced experimentally by transposon-mediated insertional inactivation of specific genes that are located on the genome [38–40]. These genes initially termed *fem* (factors essential for methicillin resistance) or *aux* (auxiliary) factors are normal constituents of the staphylococcal genome. Most of them are involved in one way or another in cell wall biosynthesis and turnover, and it is more appropriate to rename them according to their true function when it becomes known.

Contribution of the normal *S. aureus pbp2* operon to resistance

The contributions of the *mecA*-encoded PBP2′ to cell wall biosynthesis and its role in resistance are still unclear. PBP2′ shows *in vitro* transpeptidase activity [41], and seems not to contribute to cell wall biosynthesis in the absence of methicillin. In the presence of methicillin, it is rather a poor transpeptidase, able to form muropeptide dimers only [42]. The current model assumes that the low-affinity PBP2′ substitutes for the functions of the normal staphylococcal PBPs in the presence of inhibitory concentrations of β-lactams which acylate and inactivate the normal set of PBPs. This model is certainly more complex and has to be revised. Recently, inactivation of the normal PBP2 was shown to reduce methicillin resistance [43]. Full recovery of the resistance required PBP2 plus a gene included in the *pbp2* operon, coding for a protein of unknown functions [44], showing that there must be some kind of co-operation between PBP2′ and the normal PBP2.

fmt

The function of the Fmt protein (a factor which affects methicillin resistance and autolysis in the presence of Triton X-100) is unknown. It has a hydropathy pattern similar to that of *S. aureus* PBPs and contains two of three conserved motifs shared by PBPs and β-lactamases. It is thought to be involved in cell wall synthesis and causes upon inactivation a slight reduction in the molar content of the *S. aureus* peptidoglycan. It was detected as a mutation that decreases the resistance to methicillin in the presence of Triton X-100 [45]. The effect is strain dependent and may also be seen in the absence of Triton X-100 in some MRSA strains.

femX, femA-femB

The requirements of PBP2′ on its substrate are rather strict. Methicillin resistance can only be expressed in strains producing the characteristic muropeptide pentaglycine side chain which is synthesised by sequential addition of glycine residues from glycyl-t-RNAs to the muropeptide under the control of at least three proteins, FemX, FemA and FemB, which have some sequence homology to each other [reviewed in ref. 46]. The incorporation of gly2-gly3 and gly4-gly5 is controlled by FemA and FemB, respectively, encoded by the *femAB* operon [47, 70, 71]. A candidate for the attachment of gly1 by FemX has recently been identified (*fmhB*, *fem* homology factor) [48]. Reduction of the length of the glycine side chain by *femAB* inactivation leads to impaired growth, reduced cell wall turnover and peptidoglycan cross-linking, and hypersusceptibility to all β-lactams. *femAB*-null mutants need compensatory mutations to survive and also are hypersusceptible to other antibiotics [49]. The *fmhB* gene seems essential [48]. This shows that the pentaglycine side chain is an absolute requirement for PBP2′ function. Any other changes, such as reduction in the synthesis rate or the composition of the peptidoglycan precursor, also have a negative effect on methicillin resistance, but not as pronounced as the shortening of the side chain.

glnRA (*femC*)

Muropeptide precursors with a non-amidated iD-glutamate in the stem peptide take part in transpeptidation reactions less readily, leading to a reduction in methicillin resistance. This can be observed in *femC* mutants where a Tn*551* insertion in the glutamine synthetase repressor *glnR* has a polar effect on glutamine synthetase (*glnA*) transcription, leading to a shortage of glutamine that results finally in a reduced amidation of the iD-glutamate [50]. However, spontaneous methi-

cillin-resistant suppressor mutants can be selected from *femC* mutants, which are highly resistant despite the non-amidated residue, showing that this deficit can be compensated in another yet unknown fashion.

glmM (*femD*, *femR*)

One of the first steps in cytoplasmic peptidoglycan precursor formation is the interconversion of glucosamine-6-phosphate by the GlmM phosphoglucosamine mutase into glucosamine-1-phosphate [51]. Inactivation of *glmM* (mutants termed *femD* [38] or *femR* [52]) decreases the rate of peptidoglycan precursor formation and leads to reduced methicillin resistance and increased teicoplanin susceptibility [53]. GlmM is encoded by the last gene of the three-cistron *orf1-orf2-glmM* operon. As with the *glnR* mutants, highly methicillin-resistant suppressor mutants can be isolated from *glmM* mutants. The nature of the suppressor mutation is unknown, it renders the cells highly resistant to methicillin, but does not affect the teicoplanin hypersusceptibility. Both original methicillin resistance and normal teicoplanin susceptibility can be restored by complementation with the complete *orf1-orf2-glmM* operon [53], suggesting that *orf1* and *orf2* may be involved in cell wall synthesis.

murE (*femF*)

An insertion in the *murE* gene causes an accumulation of UDP-linked muramyl dipeptide and reduced levels of UDP-muramyl pentapeptide in the cytoplasmic peptidoglycan precursor pool and the incorporation of a small amount of these abnormal muropeptides into the peptidoglycan of the mutant. It is unclear here if reduction in resistance is due to the abnormal balance of peptidoglycan precursors or to the incorporation of these shortened precursors into the peptidoglycan [54].

llm

The *llm* gene codes for a lipophilic membrane protein which affects the bacterial lysis rate and methicillin resistance levels, but its function is still unknown. A Tn*918* insert in the Llm C-terminal part reduces high homogeneous methicillin resistance to a lower heteroresistant phenotype [55]. The C-terminal part of Llm is not highly conserved between different *S. aureus* isolates and some Llm activity is left in the mutant, since resistant revertants could be created by Tn*256* transposition-mediated enhancement of *llm* transcription due to the creation of a new promoter [56]. Involvement of IS-mediated stimulation of gene expression opens an interesting aspect in the control of methicillin resistance.

Lytic enzymes

Staphylococcal murein hydrolases play an important role in peptidoglycan growth and turnover [3]. In *Escherichia coli* they act in association with the PBPs, as described in the divisome model where degradation of the peptidoglycan by lytic enzymes is part of cell growth and separation [57]. For *S. aureus*, no such model has yet been established. *S. aureus* possesses multiple sets of autolytic activities [58]; however, observations of their effects on methicillin resistance have been contradictory. Whereas less autolytic activity was reported in a high-level-resistant strain [59], the opposite was described in another MRSA [60], so that there may be profound strain differences. The *atl* operon coding for two lytic enzymes involved in cell separation and β-lactam-induced lysis causes, upon inactivation, only a modest decrease in the level of methicillin resistance [61]. The first genetic evidence for a linkage of high-level resistance with a mutation in a lytic activity is the deletion of the *lytH* gene which increases resistance dramatically from low-level heterogeneous resistance to high-level homogeneous resistance [62]. This mutation seems to be one of the reasons for the occurrence of high-level subpopulations in MRSA.

Global regulators: *sar*, *agr*, *sigB*

The global regulators *sar* and *agr*, which control cell density-dependent production of cell-wall-associated and extracellular virulence factors [63], have apparently only a slight effect on methicillin resistance in heterogeneously resistant MRSA. The correlation between reduction of the maximal attainable methicillin resistance level in *agr*/*sar* double mutants and reduced PBP1 and PBP3 levels [64] led to the hypothesis that the endogenous PBPs may contribute partially to PBP2′ action, as has recently been shown for PBP2 [44]. Other factors that may be responsible for the *agr*- and *sar*-dependent resistance levels could be the modulation of the hydrolytic activities by *agr* and *sar* [65]. The P3 promoter of the *agr* operon is *sigB* dependent [66]. The stationary phase and stress-inducible transcription factor SigB [67] was shown to reduce methicillin resistance in the homogeneously highly resistant strain COL [68]. However, no *sigB*-dependent effects on methicillin resistance levels were observed in two other lineages of MRSA tested (P. Giachino and B. Berger-Bächi, unpublished results). This shows that the fine tuning of the global regulation between *agr*, *sar*, and *sigB*, is a variable strain-specific property.

ctaA

Inactivation of a *ctaA*-like open reading frame, known in *B. subtilis* to control the genetic expression of the cytochrome aa3 complex, reduced the resistance to methicillin in MRSA [69], suggesting that electron transport may have an effect on methicillin resistance. This observation needs to be analysed in further detail.

Conclusions

Methicillin resistance seems to be as complex as cell growth and separation. Apparently any factor involved in that process seems to play a role in methicillin resistance. The transposon-mediated inactivation of methicillin resistance can be exploited as a tool to identify factors involved in cell wall metabolism. The exact functions of PBP2′ in resistance are still unclear; its apparently low flexibility concerning its optimal substrate is countered by the *S. aureus* versatility in making use of compensatory mutations to optimise resistance as required. Study of the factors that influence resistance is complicated by the fact that multiple pathways may lead to a specific result, so that few generalisations can be made.

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