

Proteolysis of *mecA* Repressor Is Essential for Expression of Methicillin Resistance by *Staphylococcus aureus*

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Recently, we have demonstrated that the cognate regulatory locus of the *mecA* gene in methicillin-resistant *Staphylococcus aureus* (MRSA) is in fact a three-component system containing the novel *mecR2* gene coding for an antirepressor. MecR2 interacts with the repressor MecI, disturbing its binding to the *mecA* promoter and fostering its proteolysis. Here, we engineered a point mutation in the putative cleavage site of MecI and demonstrated that MecI proteolysis is strictly required for the optimal expression of β -lactam resistance.

In response to β -lactam chemotherapy, *Staphylococcus aureus* has acquired two resistance mechanisms: the production of a β -lactamase, coded by *blaZ*, which confers resistance to penicillins, and the production of an extra penicillin-binding protein (PBP2a), coded by *mecA*, with reduced affinity to virtually all other β -lactams. Until recently, the transcriptional control of both resistance genes was thought to be regulated by homologous two-component systems consisting of a bifunctional sensor-inducer, BlaR1 or MecR1, respectively, and a transcriptional repressor, BlaI or MecI, respectively. However, we have recently demonstrated that the *mecA* regulatory locus is in fact a three-component system that also contains the *mecR2* gene, coding for an antirepressor. MecR2 interacts directly with MecI, disturbing its binding to the *mecA* promoter and fostering its proteolysis (1). Surprisingly, in sharp contrast to what has been described for the *bla* system (2, 3), the MecI proteolysis occurs in the absence of MecR1 and, as such, must be mediated by native cytoplasmatic proteases. Here, we thought to clarify the role of MecI proteolysis in the expression of β -lactam resistance by methicillin-resistant *Staphylococcus aureus* (MRSA). For that purpose, we engineered a point mutation in the putative cleavage site of MecI, N¹⁰¹-F (4). The MecI variant was then introduced into prototype MRSA strain N315 (5), which has been used in many studies addressing the transcriptional control of *mecA* and in which we described the function of the *mecR2* gene. The phenotypic expression of β -lactam resistance, as well as the proteolysis of MecI, were then evaluated in a recombinant variant of strain N315 overexpressing the MecI variant.

To generate the MecI mutant variant (MecI^{MUT}) we used the QuikChange site-directed mutagenesis strategy (6). Briefly, using as the template DNA a recombinant high-copy-number *Escherichia coli*-*S. aureus* shuttle plasmid overexpressing a wild-type copy of *mecI*, pGC2::*mecI* (7), complementary mutagenic primer sequences (MecI-SDM1, 5' GGT TTC AAT TCA CTT GTC TTG CAG CTT GTA GAA AAA GAA GAT CTA TC 3', and MecI-SDM2, 5' GAT AGA TCT TCT TTT TCT ACA AGC TGC AAG ACA AGT GAA TTG AAA CC 3'; miss-priming positions are underlined) were extended with Phusion high-fidelity DNA polymerase (New England BioLabs). Parental methylated and hemimethylated DNA was digested with DpnI (New England BioLabs) overnight, followed by enzyme inactivation at 80°C for 20 min and dialysis through a 0.025

μ M membrane (Millipore) at 1:1,000 for 30 min. Mutated plasmid DNA was then transformed into XL10 Gold ultracompetent *E. coli* cells (Agilent Technologies). The DNA sequence of the MecI^{MUT} insert was confirmed after PCR amplification with two flanking primers (MecI-P1, 5' GC ACA ACA AAT TTC TGA GCG 3', and MecI-P2, 5' TC AAC GAC TTG ATT GTT TCC 3'). Finally, the recombinant plasmid pGC2::*mecI*^{MUT} was introduced into the restriction-deficient strain RN4220 (R. Novick) by electroporation and then transduced into the prototype MRSA strain N315, as previously described (8, 9). The phenotypic expression of β -lactam resistance was evaluated with 1-mg-oxacillin diffusion disks prepared in-house (10). The proteolysis of MecI under induction conditions (sub-MIC oxacillin concentration of 0.05 mg/liter) was analyzed by Western blotting with a polyclonal antibody raised against purified MecI (Eurogentec), as previously described (1).

In previous studies, we observed that, contrary to what was expected, the overexpression in *trans* of MecI in a representative collection of prototype MRSA strains did not cause significant effects on the expression of β -lactam resistance in most cases (7). Later, we demonstrated that the "MecI overexpression protection effect" was due to the presence of a third regulator in the *mecA* locus, MecR2, acting as a potent antirepressor and fostering the proteolysis of MecI (1). As shown by the results in Fig. 1, when prototype MRSA strain N315, which is *mecR2* positive, was transformed with the recombinant plasmid overexpressing the MecI mutant variant with a mutation in the putative cleavage site [N315(pGC2::*mecI*^{MUT})], the levels of resistance to oxacillin decreased much more substantially than when strain N315 was transformed with wild-type MecI [N315(pGC2::*mecI*)]. These observations suggest that the protective function of the anti-repressor MecR2 is lost in the presence of a MecI variant that is resistant to proteolysis. In order to further explore these observations, we have evaluated the proteolysis of MecI for the parental strain N315 and for both re-

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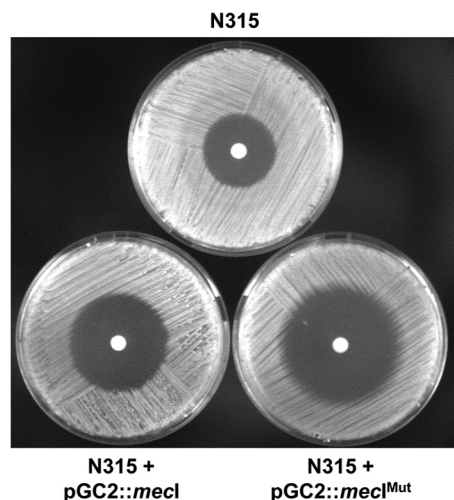


FIG 1 Analysis of the phenotypic expression of β -lactam resistance. The phenotypic expression of β -lactam resistance in prototype strain N315 and recombinant strains overexpressing wild-type and mutant MecI variants [N315(pGC2::mecI) and N315(pGC2::mecI^{MUT})] was evaluated with diffusion disks containing 1 mg of oxacillin.

combinant strains, overexpressing wild-type and mutant MecI [N315(pGC2::mecI) and N315(pGC2::mecI^{MUT})]. Previously, we have observed that the presence of MecR2 was required for the accumulation of cleaved MecI under induction conditions (1). As shown by the results in Fig. 2, in recombinant strain N315(pGC2::mecI^{MUT}), MecI accumulates in its intact form, explaining the poor expression of oxacillin resistance in this recombinant strain.

Together, these observations confirm that the proteolysis of MecI occurs at position N¹⁰¹-F and demonstrate that it is strictly necessary for the optimal induction of β -lactam resistance in MRSA strains. Therefore, similar to what has been demonstrated for the *bla* system of *S. aureus* (2), the proteolysis of the repressor protein is required for proper induction of *mecA* and is not a secondary event, as described for the homologous *pen* system of *Bacillus licheniformis* (11). However, contrary to what happens for the *bla* system (2, 3), the proteolysis of MecI is independent of the sensor-inducer MecR1 and, presumably, is mediated by native cytoplasmatic proteases (1). The elucidation of the fine molecular details of the induction mechanism underlying the expression of the *mecA* gene may pave the way for the design of alternative therapeutic strategies targeting the MRSA phenotype and, hence, contribute to attenuation of the burden of MRSA infections.

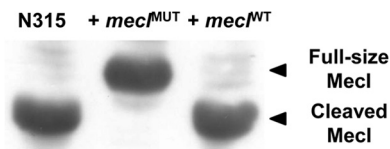


FIG 2 Analysis of the MecI proteolysis by Western blotting. The proteolysis of MecI under induction conditions (sub-MIC of oxacillin at 0.05 mg/liter) was evaluated for prototype strain N315 and recombinant strains overexpressing wild-type and mutant MecI variants [N315(pGC2::mecI) and N315(pGC2::mecI^{MUT})] using a polyclonal antibody raised against purified MecI.

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